

TITLE
ISOLATION AND EXPRESSION OF A GENE FOR A NITRILASE
FROM *ACIDOVORAX FACILIS* 72W

FIELD OF INVENTION

5 The present invention relates to the field of molecular biology and the use of recombinant microorganisms to express desired genes and gene products. More specifically, the gene used in the invention is a novel nucleic acid fragment encoding a nitrilase enzyme that catalyzes the hydrolysis of a wide variety of nitrile-containing substances to produce a corresponding carboxylic acid. Also provided are recombinant strains that express nitrilase activity and are useful as biocatalysts for the hydrolysis of nitrile-containing substrates. Additionally, the invention relates to specific nucleic acids that aid in isolating such nitrilase genes.

BACKGROUND

15 Nitriles are readily converted to the corresponding carboxylic acids by a variety of chemical processes, but these processes typically require strongly acidic or basic reaction conditions and high reaction temperatures, and usually produce unwanted byproducts and/or large amounts of inorganic salts as unwanted waste. Processes in which enzyme-catalyzed hydrolysis converts nitrile-containing substrates to the corresponding carboxylic acids are often preferred to chemical methods because these processes 1) are often run at ambient temperature, 2) do not require the use of strongly acidic or basic reaction conditions, and 3) do not produce large amounts of unwanted byproducts. Especially advantageous over chemical hydrolysis, the enzyme-catalyzed hydrolysis of a variety of aliphatic or aromatic dinitriles can be highly regioselective, where only one of the two nitrile groups is hydrolyzed to the corresponding carboxylic acid ammonium salt.

Enzyme-catalyzed hydrolysis of nitrile substrates to the corresponding carboxylic acids may be accomplished via a one- or two- step reaction (Table 1).

TABLE 1

	Substrates*	Product(s)	Enzyme
Two Step			
Reaction 1	$\text{RCN} + \text{H}_2\text{O}$	RC(O)NH_2	nitrile hydratase
Reaction 2	$\text{RC(O)NH}_2 + \text{H}_2\text{O}$	$\text{RC(O)OH} + \text{NH}_3$	amidase
One Step			
Reaction 1	$\text{RCN} + 2\text{H}_2\text{O}$	$\text{RC(O)OH} + \text{NH}_3$	nitrilase

*R represents a varying spectrum of organic substituents particular to a chosen enzyme.

A wide variety of bacterial genera collectively possess a diverse spectrum of nitrile hydratase, amidase, or nitrilase activities, including *Rhodococcus*,

Pseudomonas, *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Bacteridium*, *Brevibacterium*, *Corynebacterium*, *Agrobacterium*, *Micrococcus*, and *Comamonas*. Both aqueous suspensions of these microorganisms and the isolated enzymes have been used to convert nitriles to carboxylic acids. Biotechnological use of these enzymes has been recently reviewed by Cowan et al. (*Extremophiles* (1998) 2:207-216).

A nitrilase enzyme directly converts a nitrile to the corresponding carboxylic acid in aqueous solution without the intermediate formation of an amide. The use of nitrilases for the hydrolysis of aromatic nitriles to the corresponding carboxylic acid ammonium salts has been known for many years, but it is only recently that the use of nitrilases to convert aliphatic nitriles has been reported. Kobayashi et al. (*Tetrahedron* (1990) 46:5587-5590; *J. Bacteriology* (1990) 172:4807-4815) have described an aliphatic nitrilase isolated from *Rhodococcus rhodochrous* K22 which catalyzed the hydrolysis of aliphatic nitriles to the corresponding carboxylic acid ammonium salts; several aliphatic α,ω -dinitriles were also hydrolyzed. A nitrilase from *Comamonas testosteroni* has been isolated which can convert a range of aliphatic α,ω -dinitriles to either the corresponding ω -cyanocarboxylic acid ammonium salt or the dicarboxylic acid diammonium salt (CA 2,103,616; and Lévy-Schil et al., *Gene* (1995) 161:15-20).

The nitrilase activity of unimmobilized *Acidovorax facilis* 72W cells has been used in a process to prepare five-membered or six-membered ring lactams from aliphatic α,ω -dinitriles (US 5,858,736). In that process, an aliphatic α,ω -dinitrile is first converted to an ammonium salt of an ω -cyanocarboxylic acid in aqueous solution using a catalyst having an aliphatic nitrilase (EC 3.5.5.7) activity. The ammonium salt of the ω -cyanocarboxylic acid is then converted directly to the corresponding lactam by hydrogenation in aqueous solution, without isolation of the intermediate ω -cyanocarboxylic acid or ω -amino-carboxylic acid. When the aliphatic α,ω -dinitrile is also unsymmetrically substituted at the α -carbon atom, the nitrilase produces the ω -cyanocarboxylic acid ammonium salt resulting from hydrolysis of the ω -nitrile group with greater than 98 % regioselectivity, thereby producing only one of the two possible lactam products during the subsequent hydrogenation. For example, 2-methyl-glutaronitrile (MGN) was hydrolyzed by unimmobilized *Acidovorax facilis* 72W cells to produce 4-cyanopentanoic acid (4-CPA) ammonium salt with greater than 98 % regioselectivity at 100 % conversion.

Nitrilase genes have been cloned and expressed in heterologous systems, especially in *Escherichia coli*. Petre et al. (US 5,635,391) disclose expression of a nitrilase from *Comamonas testosteroni* in *E. coli* and *Pseudomonas putida*. Also

disclosed is a method to improve levels of soluble nitrilase protein in *E. coli* by coexpression of the GroE chaperonin protein, which results in higher nitrilase specific activity. The *E. coli* promoters P_{lac} and P_{trp} were used to drive expression of the nitrilase coding sequences. In *E. coli* P_{lac} has also been used successfully in expressing nitrilase coding sequences from *Alcaligenes faecalis* JM3 (Kobayashi et al., *Proc. Nat. Acad. Sci.* (1993) 90:247 and JP #4-30663), *Rhodococcus rhodochrous* J1 (Kobayashi et al., *J. Biol. Chem.* (1992) 267:20746) and *Rhodococcus rhodochrous* K22 (Kobayashi et al., *Biochem.* (1992) 31, 9000). Stalker (US 4,810,648) discloses that the gene for a haloarylnitrile-hydrolyzing nitrilase can be expressed under control of its native promoter in *E. coli*. In US 5,602,014, Mizumura and Yu disclose a specialized regulatory system for expression of nitrilase genes in *Rhodococcus erythropolis*.

Nitrilase enzymes are reported to be highly labile and not obtainable in large quantities (Kobayashi et al., *Tetrahedron* (1990) 46:5587-5590; *J. Bacteriology* (1990) 172:4807-4815). In contrast to nitrile hydratase, nitrilases are characterized by low specific activities and reaction rates (Nagasawa et al., *Appl Microbiol. Biotechnol.* (1993) 40:189-195). The inherent thermal instability of nitrile-hydrolyzing enzymes from mesophiles is reported to limit their industrial applications (Cramp et al., *Microbiol.* (1997) 143:2313-2320).

The problem remains the lack of an industrially useful, thermostable, and highly productive nitrilase enzyme suitable as a catalyst for nitrile-containing substrates in applications (such as the regioselective hydrolysis of aliphatic dinitriles to cyanocarboxylic acids) where high yields of product are obtained under mild reaction conditions (including ambient temperatures and without extreme acidic or basic conditions) and without generating relatively large amounts of undesirable wastes.

SUMMARY OF THE INVENTION

The instant invention provides for an isolated DNA sequence specific to the *Acidovorax* 72W nitrilase gene. The instant invention includes isolated nucleic acid fragments that are complementary to the complete sequences of the accompanying Sequence Listing as well as substantially similar nucleic acid sequences. The instant invention includes any nucleic acid fragment that encodes all or a substantial portion of the amino acid sequence of the nitrilase enzyme as set forth in SEQ ID NO:5 or SEQ ID NO:14. The invention also includes an isolated nucleic acid molecule that hybridizes with the nucleic acid fragment encoding all or a substantial portion of the amino acid sequences of SEQ ID NO:5 or SEQ ID NO:14 under hybridization conditions of 6 x SSC (1M NaCl), 40-45 % formamide, 1 % SDS at 37 °C, and a wash in 0.5x to 1x SSC at 55 to 60 °C.

Nucleic acid fragments complementary to the listed SEQ ID NOs:1-16 are also claimed. Polypeptides encoded by the nucleic acid fragments of the invention are also provided, for example, SEQ ID NO:5 and SEQ ID NO:14. The invention includes RNA or antisense RNA molecules transcribed from sequences described above and their cDNA derivatives.

Also provided are methods to produce active enzyme protein from these nitrilase coding sequences in transformed microorganisms. The improvement obtained from this invention is an increased reaction rate and higher reactor productivity due to the increased specific activity of the transformant catalysts relative to *Acidovorax facilis* 72W. The active nitrilase enzyme produced in whole cells transformed with genetic materials described herein can be used to carry out useful hydrolysis of nitrile-containing substrates. Transformed microorganisms containing expression cassettes comprising chimeric genes having plasmids containing the isolated nucleic acid fragment encoding nitrilase enzyme, each unit as described herein, are also part of this invention. One embodiment uses any of the specific enzyme catalysts *E. coli* SW91 (ATTC PTA-1175), *E. coli* DH5 α :pnit4 (ATTC PTA-1176), *E. coli* SS1001 (ATTC PTA-1177), *E. coli* SS1002 containing plasmid pnitex2, or *E. coli* SS1011 containing plasmid pnitex2 in contact with α,ω -cyanocarboxylic acid. A further embodiment uses the specific transformed microorganisms in an improved method to convert α,ω -dinitriles to ω -cyanocarboxylic acids, which are intermediate in the preparation of five-membered or six-membered ring lactams (See U.S. 5,858,736).

A method is provided for using a native microbial gene, specifically from *Acidovorax facilis* 72W, encoding a protein characterized by a nitrilase activity on nitrile-containing substrates to obtain a mutated microbial gene encoding a protein characterized by an increased specific nitrilase activity on nitrile-containing substrates and/or increased stability of the nitrilase, one or both characteristics increased relative to that of the native microbial gene, the mutated microbial gene produced by a method comprising the steps of

- (i) contacting restriction endonucleases with a mixture of nucleotide sequences to yield a mixture of restriction fragments, the mixture of nucleotide sequences comprising
 - a) a native microbial gene;
 - b) a first population of nucleotide fragments which will hybridize with the nucleotide sequences of the native microbial gene of (i)(a); and

- c) a second population of nucleotide fragments which will not hybridize to the nucleotide sequences of the native microbial gene of (i)(a),
- (ii) denaturing the mixture of restriction fragments of step (i);
- (iii) incubating the denatured mixture of restriction fragments of step (ii) with a polymerase; and
- (iv) repeating steps (ii) and (iii) a sufficient number of times to yield a mutated microbial gene encoding a protein characterized by an increased specific nitrilase activity on nitrile-containing substrates and/or increased stability of the nitrilase, on or both characteristics increased relative to that of the native microbial gene. The invention includes mutated microbial genes produced by this method.

BRIEF DESCRIPTION OF THE FIGURES, BIOLOGICAL DEPOSITS, AND SEQUENCE LISTING

The invention can be more fully understood from the figure, the biological deposits, the accompanying sequence descriptions, the detailed description, and claims which together constitute this application.

Figure 1 shows a restriction map of the 4.1 kb *Pst*I fragment of pnit4. The *Bcl*II-*Bgl*II fragment contains the nitrilase gene and the flanking chromosomal region.

Applicants have made the following biological deposits under the terms of the Budapest Treaty on the International Recognition of the Deposit of Micro-organisms for the purposes of Patent Procedure:

Depositor Identification Reference	International Depository Designation	Date of Deposit
<i>E. coli</i> SW91	ATCC PTA-1175	11 January 2000
<i>E. coli</i> DH5 α : pnit4	ATCC PTA-1176	11 January 2000
<i>E. coli</i> SS1001	ATCC PTA-1177	11 January 2000

As used herein, "ATCC" refers to the American Type Culture Collection International Depository Authority located at ATCC, 10801 University Blvd., Manassas, VA 20110-2209, USA. The "International Depository Designation" is the accession number of the culture on deposit with ATCC.

The listed deposit(s) will be maintained in the indicated international depository for at least thirty (30) years and will be made available to the public upon the grant of a patent disclosing it. The availability of a deposit does not

constitute a license to practice the subject invention in derogation of patent rights granted by government action.

Applicant(s) have provided 32 sequences in conformity with 37 C.F.R. 1.821-1.825 ("Requirements for Patent Applications Containing Nucleotide Sequences and/or Amino Acid Sequence Disclosures - the Sequence Rules") and consistent with World Intellectual Property Organization (WIPO) Standard ST.25 (1998) and the sequence listing requirements of the EPO and PCT (Rules 5.2 and 49.5(a-bis), and Section 208 and Annex C of the Administrative Instructions). The symbols and format used for nucleotide and amino acid sequence data comply with the rules set forth in 37 C.F.R. §1.822.

SEQ ID NO:1 is the nucleotide sequence of a forward primer (1F) which was derived from a conserved region found in bacterial nitrilase sequences available from GenBank data and was used as degenerate PCR primers to discover a novel nitrilase gene.

SEQ ID NO:2 is the nucleotide sequence of a reverse primer (7R) which was derived from a conserved region found in bacterial nitrilase sequences available from GenBank data and was used as degenerate PCR primers to discover a novel nitrilase gene.

SEQ ID NO:3 is the nucleotide sequence of the *Acidovorax facilis* 72W genomic portion of the pJJ28-5 clone without degenerate primer regions.

SEQ ID NO:4 is the nucleotide sequence of the *Acidovorax facilis* 72W nitrilase coding sequence identified within the 4.1 kb insert from pnit4.

SEQ ID NO:5 is the amino acid sequence deduced from the nucleotide sequence (SEQ ID NO:4, SEQ ID NO:15) encoded by *Acidovorax facilis* 72W nitrilase coding sequence.

SEQ ID NO:6 is the nucleotide sequence of a forward primer which was used to amplify the nitrilase coding sequence from *Acidovorax facilis* 72W genomic DNA.

SEQ ID NO:7 is the nucleotide sequence of a reverse primer which was used to amplify the nitrilase coding sequence from *Acidovorax facilis* 72W genomic DNA.

SEQ ID NO:8 is the nucleotide sequence of a forward primer which was used to amplify the nitrilase coding sequence from *Acidovorax facilis* 72W genomic DNA.

SEQ ID NO:9 is the nucleotide sequence of a reverse primer which was used to amplify the nitrilase coding sequence from *Acidovorax facilis* 72W genomic DNA.

SEQ ID NO:10 is the nucleotide sequence of a forward primer which was used to amplify the nitrilase coding sequence from *Acidovorax facilis* 72W genomic DNA.

5 SEQ ID NO:11 is the nucleotide sequence of a forward primer which was used to amplify the nitrilase coding sequence from *Acidovorax facilis* 72W genomic DNA and which was also used to incorporate *XhoI* restriction sites.

SEQ ID NO:12 is the nucleotide sequence of a reverse primer used to amplify the nitrilase coding sequence from *Acidovorax facilis* 72W genomic DNA and which was used to incorporate *XhoI* restriction sites.

10 SEQ ID NO:13 is the nucleotide sequence of the nitrilase coding sequence in plasmid pnitex2 used for overexpression in *E. coli*.

SEQ ID NO:14 is the deduced amino acid sequence encoded by the nitrilase coding sequence in plasmid pnitex2 used for overexpression of nitrilase enzyme in *E. coli*.

15 SEQ ID NO:15 is the nucleotide sequence of a 1776 bp *BclI*-*BglII* genomic fragment of the plasmid pnit4 containing nitrilase gene and flanking chromosomal region from *Acidovorax facilis* 72W.

SEQ ID NO:16 is a synthetic version of the nitrilase gene, with codon usage optimized for expression in *Pichia*.

20 SEQ ID NOs:17-32 are oligonucleotides used to construct the synthetic nitrilase gene of SEQ ID NO:16.

DETAILED DESCRIPTION OF THE INVENTION

Applicants have solved the stated problem with an invention relating to the use of a nitrilase gene sequence encoding a protein useful as a biocatalyst for the hydrolysis of nitrile-containing substrates to a carboxylic acid. The product of nitrile hydrolysis, a carboxylic acid, may also be in the form of a carboxylic acid salt with byproducts of ammonia or other buffer components. The nitrilase gene was isolated from *Acidovorax facilis* 72W. Specifically, recombinant strains of host cells (such as *E. coli*) that express active *Acidovorax facilis* 72W nitrilase protein are extremely useful to catalyze the hydrolysis of a wide variety of nitrile-containing substrates, including the highly regioselective hydrolysis of dinitriles. Typical dinitriles will have the formula NC-R-CN where R is an alkylene group having from about 1 to about 10 carbons.

35 This biocatalytic process is industrially attractive as it generates much smaller quantities of undesirable wastes and operates under much milder conditions than previously known methods. The products of the present invention are useful as precursors for polymers, solvents, and chemicals of high value in the chemical, agricultural, and pharmaceutical industries.

Applicants' solution to the problems existing in the technical field arose out of the following accomplishments which are discussed in more detail below and in the Examples:

- I. provided degenerate PCR primer sequences (SEQ ID NOs:1 and 2) useful for identifying the presence of unknown nitrilase genes in bacteria;
 - II. isolated nucleic acid sequences useful in screening for the presence of any nitrilase gene;
 - III. mapped, identified, and cloned the complete coding sequence for a regioselective, thermostable nitrilase from *Acidovorax facilis* 72W (ATCC 55746) (SEQ ID NO:4 and 15);
 - IV. constructed recombinant DNA plasmids pSW91, pnit4, and pnitex2, chimeric genes, and expression cassettes containing the coding sequence as described in III located within a 4.1 kb chromosomal DNA fragment from *Acidovorax facilis* 72W (ATCC 55746);
 - V. constructed recombinant strains of *E. coli* that express active *Acidovorax facilis* 72W nitrilase protein;
 - VI. demonstrated regioselective hydrolysis of 2-methylglutaronitrile (MGN) to 4-cyanopentanoic acid (4-CPA) using whole cells of the recombinant *E. coli* expressing the *Acidovorax facilis* 72W nitrilase protein described herein; and teach the use of a native microbial gene encoding a protein characterized by a nitrilase activity on nitrile-containing substrates (preferably 2-methylglutaronitrile) to obtain a mutated microbial gene encoding a protein characterized by altered nitrilase activity and/or greater stability, one or both characteristic increased relative to that of the native nitrilase protein.
- Acidovorax facilis* 72W nitrilase proved to be an unexpectedly robust catalyst for producing carboxylic acids from aliphatic or aromatic nitriles. All known nitrilases, including *Acidovorax facilis* 72W nitrilase, have a nucleophilic cysteine in the enzyme active site (Cowan et al., (1998) *supra*) and all are susceptible to inactivation by thiol reagents (1.0 mM concentrations of copper chloride, silver nitrate, mercuric acetate, or ferric chloride each produced major decreases in 72W nitrilase enzyme activity). Cysteine residues are also capable of being irreversibly oxidized to sulfinic acids, resulting in a loss of enzyme activity. Despite the sensitivity of nitrilase enzymes to various inactivating mechanisms, immobilized *Acidovorax facilis* 72W cells used as a catalyst produced up to 3.9×10^7 moles of product (4-CPA) per mole of nitrilase enzyme (total turnover number, TTN).

The expression of active *Acidovorax facilis* 72W nitrilase in a heterologous host cell has several additional advantages over the preparation and use of

Acidovorax facilis 72W cells as a nitrilase catalyst. The level of expression of the nitrilase protein in *Acidovorax facilis* 72W is ca. 3.4 % of total soluble protein (Example 14) and is constitutive; a thorough screening of potential inducers of nitrilase production (aliphatic or aromatic nitriles or amides, lactams, ureas, etc.) found no effect on the level of nitrilase produced during fermentation of *Acidovorax facilis* 72W.

In contrast, *E. coli* transformants expressed enzymatically-active *Acidovorax facilis* 72W nitrilase at levels of up to 12 % of total soluble protein; the total amount of nitrilase produced (active and inactive) in *E. coli* transformants was as high as 58 % of total soluble protein. This increased level of nitrilase expression in *E. coli* transformant cells results in up to a 2.4-fold increase in specific activity (units of nitrilase activity per gram dry cell weight) relative to *Acidovorax facilis* 72W cells, which in turn significantly increases the catalyst productivity (g product produced/g dry cell weight/h) (Examples 7 and 9).

Moreover, *Acidovorax facilis* 72W cells require glycerol, a relatively expensive carbon substrate, when grown by fermentation, and have not been successfully grown using inexpensive glucose. In contrast, *E. coli* transformants can be grown on glucose to the same cell density as *Acidovorax facilis* 72W cells in about half the time, significantly reducing biocatalyst production costs.

Acidovorax facilis 72W cells additionally contain a nitrile hydratase and an amidase, neither of which are regioselective, and which can produce unwanted byproducts when converting α,ω -dinitriles to the corresponding ω -cyanocarboxylic acid ammonium salts. *Acidovorax facilis* 72W cells required heat-treatment to inactivate the nitrile hydratase and amidase enzymes (US 5,814,508), risking loss of nitrilase activity and adding production costs. *Acidovorax facilis* mutant strains 72-PF-15 and 72-PF-17 were prepared which lacked the nitrile hydratase enzyme (US 5,858,736), but these strains had only ca. half the nitrilase specific activity of the parent *Acidovorax facilis* 72W strain.

In contrast, an *E. coli* transformed with the genetic material to express nitrilase enzyme does not require a heat-treatment step to inactivate unwanted nitrile hydratase or amidase activities, eliminating the cost of this processing step for catalyst production and avoiding the inadvertent loss of nitrilase activity.

E. coli transformants having a high specific nitrilase activity with the attendant advantages have been produced by Applicants as described in Examples 6 and 7.

The whole-cell nitrilase activity of *Acidovorax facilis* 72W is very stable at temperatures of up to about 55 °C; a cell suspension in 0.10 M phosphate buffer (pH 7.0) had a nitrilase half-life of 22.7 h at 50 °C (Gavagan et al., *Appl. Microbiol. Biotechnol.* (1999) 52:654-659). The purified enzyme also had

excellent temperature stability, where no loss of activity of a 10 mg/mL solution of purified nitrilase in 0.10 M phosphate buffer was observed after 24 h at 45 °C. When stored as a solution in 50 mM potassium phosphate buffer (pH 7.0) at 5 °C, no loss of activity of purified nitrilase was observed after 46 days. Although

5 *Acidovorax facilis* 72W, a mesophilic bacterium, has an optimal temperature for growth of 32 °C, the 72W nitrilase enzyme itself has a thermal stability that compares favorably to the nitrilase enzyme of a thermophilic bacterium such as the *Bacillus pallidus* strain DAC521 (Cramp et al., *Microbiol.* (1997) 13:2313-2320).

10 When unimmobilized cells are used as a catalyst for hydrolysis of MGN to 4-CPA, either centrifugation or ultrafiltration was required to recover the unimmobilized cells for reuse. At high product concentrations (up to 29 wt. % 4-CPA ammonium salt), the unimmobilized cells lost significant activity with each reuse and cell lysis was observed. In contrast, immobilizing the cells
15 simplified catalyst recovery and reuse, improved the resistance of cells to lysis, and increased the stability of the enzyme activity of the immobilized cells when recycled in consecutive batch reactions as compared to using unimmobilized cells.

Immobilization of either whole cell *Acidovorax facilis* 72W or the *E. coli* transformant SS1001 in carrageenan under identical conditions produced gels that
20 were stable when recycled in consecutive batch reactions which produced high concentrations of 4-CPA ammonium salt (up to ca. 200 g/L). Gel beads were prepared by first dispersing a heated aqueous suspension of cells (5 % dry cell weight) and carrageenan (3 wt%) in heated soybean oil at 50 °C, and the resulting droplets were gelled by lowering the temperature of the oil below the gelling
25 temperature of the carrageenan (Audet et al., *Process Biochem.* (1989) 24:217). The cell/carrageenan beads, which had an average diameter of from 0.5 mm-3 mm, were separated from the soybean oil, then washed with aqueous buffer and crosslinked with glutaraldehyde and polyethyleneimine.

Acidovorax facilis 72W cells and the *E. coli* transformant SS1001 cells
30 immobilized in carrageenan beads were compared in side-by-side reactions under identical conditions for the production of 1.25 M 4-CPA ammonium salt. The reaction rate when using immobilized *E. coli* transformant SS1001 was 1.7 times greater than when using immobilized *Acidovorax facilis* 72W cells (310 mM 4-CPA ammonium salt/h and 184 mM 4-CPA ammonium salt/h, respectively;
35 Example 9). The increase in reaction rate obtained with *E. coli* transformant SS1001 was a result of the higher specific activity (units of nitrilase activity per gram of beads) of the immobilized transformant cell catalyst, and demonstrates one advantage of using a transformant cell with higher specific nitrilase activity

relative to the parent *Acidovorax facilis* 72W strain. The reaction rate when using *E. coli* transformant SW91 cells immobilized in calcium alginate beads according to a published procedure (Bucke, *Methods Enzymol.* (1987) 135:175-189) was 4.0 times greater than when using carrageenan-immobilized *Acidovorax facilis* 72W cells (739 mM 4-CPA ammonium salt/h and 184 mM 4-CPA ammonium salt/h, respectively; Examples 9 and 11). The increased catalyst productivity (g product/g catalyst/h) achieved by using the immobilized transformant catalyst significantly reduces process costs.

The invention provides a new nucleic acid sequence encoding nitrilase enzyme. This sequence comprises an open reading frame (ORF) residing on a 4.1 kb *Pst*I fragment isolated from *Acidovorax facilis* 72W genomic DNA. The newly defined ORF encodes an identifiable enzyme that converts nitrile to the corresponding carboxylic acid ammonium salt. The ORF was identified both on the basis of expression of active nitrilase as well as comparison of the nucleic acid and deduced amino acid sequences to public databases using algorithms well known in the art. The protein coded for by the ORF showed up to a 71 % primary amino acid sequence identity to other known nitrilases.

Accordingly, preferred polypeptides of the instant invention are those active proteins that are at least 80 % identical to the amino acid sequence reported herein. More preferred amino acid fragments are at least 90 % identical to the sequences herein. Most preferred are amino acid fragments that are at least 95 % identical to the amino acid fragments reported herein. Similarly, preferred nucleic acid sequences corresponding to the instant ORF are those encoding active proteins and which are at least 80 % identical to the nucleic acid sequences reported herein. More preferred nucleic acid fragments are at least 90 % identical to the sequences herein. Most preferred are nucleic acid fragments that are at least 95 % identical to the nucleic acid fragments reported herein.

The nucleic acid fragments of the instant invention may be used to isolate cDNAs and genes encoding homologous enzymes from the same or other bacterial species. Isolation of homologous genes using sequence-dependent protocols is well known in the art. Examples of sequence-dependent protocols include, but are not limited to, methods of nucleic acid hybridization, and methods of DNA and RNA amplification as exemplified by various uses of nucleic acid amplification technologies (e.g., polymerase chain reaction, ligase chain reaction).

In general, a sequence of ten or more contiguous amino acids or thirty or more nucleotides is necessary to putatively identify a polypeptide or nucleic acid sequence as homologous to a known protein or gene. Moreover, with respect to nucleotide sequences, gene-specific oligonucleotide probes comprising

20-30 contiguous nucleotides may be used in sequence-dependent methods of gene identification (e.g., Southern hybridization) and isolation (e.g., *in situ* hybridization of bacterial colonies or bacteriophage plaques). In addition, short oligonucleotides of 12-15 bases may be used as amplification primers in PCR in order to obtain a particular nucleic acid fragment comprising the primers.

For example, genes encoding similar enzymes to that of the instant invention, either as cDNAs or genomic DNAs, could be isolated directly by using all or a portion of the instant nucleic acid fragments as DNA hybridization probes to screen libraries from any desired bacteria using methodology well known to those skilled in the art. Specific oligonucleotide probes based upon the instant nucleic acid sequences can be designed and synthesized by methods known in the art (Sambrook, J., Fritsch, E. F. and Maniatis, T., *Molecular Cloning: A Laboratory Manual* (1989), Cold Spring Harbor Laboratory Press, Cold Spring Harbor (referred to throughout as "Maniatis"). Moreover, the entire sequences can be used directly to synthesize DNA probes by methods known to the skilled artisan such as random primers DNA labeling, nick translation, or end-labeling techniques, or RNA probes using available *in vitro* transcription systems. In addition, specific primers can be designed and used to amplify a part of or full-length of the instant sequences. The resulting amplification products can be labeled directly during amplification reactions or labeled after amplification reactions, and used as probes to isolate full length cDNA or genomic fragments under conditions of appropriate stringency.

Two short segments of the instant ORF may be used in polymerase chain reaction protocols to amplify longer nucleic acid fragments encoding homologous genes from DNA or RNA. Alternatively, the second primer sequence may be based upon sequences derived from the cloning vector. For example, the skilled artisan can follow the RACE protocol to generate cDNAs by using PCR to amplify copies of the region between a single point in the transcript and the 3' or 5' end. Primers oriented in the 3' and 5' directions can be designed from the instant sequences. Using commercially available 3' RACE or 5' RACE systems (BRL), specific 3' or 5' cDNA fragments can be isolated (Ohara et al., *PNAS USA* (1989) 86:5673; Loh et al., *Science* (1989) 243:217).

Typically, in PCR-type amplification techniques, the primers have different sequences and are not complementary to each other. Depending on the desired test conditions, the sequences of the primers should be designed to provide for both efficient and faithful replication of the target nucleic acid. Methods of PCR primer design are common and well known in the art. (Thein and Wallace, "The use of oligonucleotide as specific hybridization probes in the diagnosis of

genetic disorders" (1986) pp. 33-50, in *Human Genetic Diseases: A Practical Approach*, K. E. Davis (Ed.), IRL Press, Herndon, Virginia); Rychlik, W., PCR Protocols: Current Methods and Applications. (1993) 15:31-39, in *Methods in Molecular Biology*, B.A. White, (Ed.), Humana Press, Inc., Totowa, NJ.)

5 Alternatively, the instant sequences may be used as hybridization reagents for the identification of homologs. The basic components of a nucleic acid hybridization test include a probe, a sample suspected of containing the gene or gene fragment of interest, and a specific hybridization method. Probes of the present invention are typically single stranded nucleic acid sequences which are
10 complementary to the nucleic acid sequences to be detected. Probes are "hybridizable" to the nucleic acid sequence to be detected (cDNA, genomic DNA or RNA). The probe length can vary from 5 bases to tens of thousands of bases, the length depending upon the specific test to be done. Only part of the probe molecule need be complementary to the nucleic acid sequence to be detected. In
15 addition, the complementarity between the probe and the target sequence need not be perfect. Hybridization does occur between imperfectly complementary molecules with the result that a certain fraction of the bases in the hybridized region are not paired with the proper complementary base.

 Hybridization methods are well defined (Maniatis, particularly Chapter 11
20 and Table 11.1). Typically, the probe and sample must be mixed under conditions which will permit nucleic acid hybridization. This involves contacting the probe and sample in the presence of an inorganic or organic salt under appropriate temperature and ionic strength conditions. The probe and sample nucleic acids must be in contact for a long enough time that any possible hybridization between
25 the probe and sample nucleic acid may occur. The concentration of probe or target in the mixture will determine the time necessary for hybridization to occur. The greater the probe or target concentration, the shorter the hybridization incubation time needed. Optionally, a chaotropic agent may be added. The chaotropic agent stabilizes nucleic acids by inhibiting nuclease activity.
30 Furthermore, the chaotropic agent allows sensitive and stringent hybridization of short oligonucleotide probes at room temperature (Van Ness et al., *Nucl. Acids Res.* (1991) 19:5143-5151). Suitable chaotropic agents include guanidinium chloride, guanidinium thiocyanate, sodium thiocyanate, lithium tetrachloroacetate, sodium perchlorate, rubidium tetrachloroacetate, potassium iodide, and cesium
35 trifluoroacetate, among others. Typically, the chaotropic agent will be present at a final concentration of about 3M. If desired, one can add formamide to the hybridization mixture, typically 30-50 % (v/v).

Various hybridization solutions can be used. Typically, these comprise from about 20 to 60 % volume, preferably 30 %, of a polar organic solvent. A common hybridization solution employs about 30-50 % v/v formamide, about 0.15 to 1M sodium chloride, about 0.05 to 0.1M buffers, such as sodium citrate, Tris-HCl, PIPES or HEPES (pH range about 6-9), about 0.05 to 0.2 % detergent, such as sodium dodecylsulfate, or between 0.5-20 mM EDTA, FICOLL (Pharmacia Inc.) (about 300-500 kilodaltons), polyvinylpyrrolidone (about 250-500 kilodaltons), and serum albumin. Also included in the typical hybridization solution will be unlabeled carrier nucleic acids from about 0.1 to 5 mg/mL, fragmented nucleic DNA, e.g., calf thymus or salmon sperm DNA, or yeast RNA, and optionally from about 0.5 to 2 % wt./vol. glycine. Other additives may also be included, such as volume exclusion agents which include a variety of polar water-soluble or swellable agents, such as polyethylene glycol, anionic polymers such as polyacrylate or polymethylacrylate, and anionic saccharidic polymers, such as dextran sulfate.

Nucleic acid hybridization is adaptable to a variety of assay formats. One of the most suitable is the sandwich assay format. The sandwich assay is particularly adaptable to hybridization under non-denaturing conditions. A primary component of a sandwich-type assay is a solid support. The solid support has adsorbed to it or covalently coupled to it immobilized nucleic acid probe that is unlabeled and complementary to one portion of the sequence.

Availability of the instant nucleotide and deduced amino acid sequences facilitates immunological screening of cDNA expression libraries. Synthetic peptides representing portions of the instant amino acid sequences may be synthesized. These peptides can be used to immunize animals to produce polyclonal or monoclonal antibodies with specificity for peptides or proteins comprising the amino acid sequences. These antibodies can be then be used to screen cDNA expression libraries to isolate full-length cDNA clones of interest (Lerner, *Adv. Immunol.* 36:1 (1984); and Maniatis).

Heterologous Host Cells:

The active *Acidovorax facilis* 72W nitrilase protein may be produced in heterologous host cells, preferably in microbial hosts. Particularly useful in the present invention will be cells that are readily adaptable to large-scale fermentation methods. Such organisms are well known in the art of industrial bioprocessing, examples of which may be found in "Recombinant Microbes for Industrial and Agricultural Applications", Murooka et al., eds., Marcel Dekker, Inc., New York, NY (1994), and include fermentative bacteria as well as yeast and filamentous fungi. Host cells may include but are not limited to *Comamonas sp.*,

Corynebacterium sp., *Brevibacterium sp.*, *Rhodococcus sp.*, *Azotobacter sp.*,
Citrobacter sp., *Enterobacter sp.*, *Clostridium sp.*, *Klebsiella sp.*, *Salmonella sp.*,
Lactobacillus sp., *Aspergillus sp.*, *Saccharomyces sp.*, *Zygosaccharomyces sp.*,
Pichia sp., *Kluyveromyces sp.*, *Candida sp.*, *Hansenula sp.*, *Dunaliella sp.*,
5 *Debaryomyces sp.*, *Mucor sp.*, *Torulopsis sp.*, *Methylobacteria sp.*, *Bacillus sp.*,
Escherichia sp., *Pseudomonas sp.*, *Rhizobium sp.*, and *Streptomyces sp.*

Particularly preferred is *E. coli*. Examples of suitable *E. coli* host cells in which a
nitrilase gene can be expressed include but are not limited to host cells specified
herein and MG1655 (ATCC 47076), W3110 (ATCC 27325), MC4100
10 (ATCC 35695), W1485 (ATCC 12435), and their derivatives.

Microbial Expression Systems and Expression Vectors:

Microbial expression systems and expression vectors containing regulatory
sequences that direct high level expression of foreign proteins are well known to
those skilled in the art. These could be used to construct chimeric genes for
15 production of the gene products of the 4.1 kb fragment of the invention. These
chimeric genes could then be introduced into appropriate microorganisms via
transformation to provide high level expression of the nitrilase enzymes. The
nucleotides of the present invention may be used to produce gene products having
enhanced or altered activity levels relative to that of the native gene sequence.

20 Additionally, chimeric genes will be effective in altering the properties of a
host cell. For example, introducing at least one copy of chimeric genes encoding
the present ORF under the control of the appropriate promoters into a host cell
gives the host cell the ability to convert 2-methylglutaronitrile to 4-cyanopentanoic
acid. The chimeric genes of the instant invention will comprise suitable regulatory
25 sequences useful for driving gene expression of the present nitrilase sequences.
Regulatory sequences will include, but are not limited to promoters, translation
leader sequences, and ribosomal binding sites. It is preferred if these sequences
are derived from the host organism; however, the skilled person will recognize
that heterologous regulatory sequences may also be used.

30 In one embodiment, the regulatory sequences will include a promoter.
Promoters may be constitutive or inducible. Inducible promoters are generally
responsive to a specific stimulus (e.g., IPTG inducing the *lac* promoter).
Inducible promoters may be responsive to a variety of stimuli, including,
chemicals, growth cycle, changes in temperature, changes in pH and changes in
35 osmolarity, to name only a few. In a preferred embodiment of the invention it has
been discovered that a chimeric gene comprising a nitrilase coding regions (e.g.,
SEQ ID NO:4, SEQ ID NO:13 OR SEQ ID NO:15) is expressed as effectively, or

more effectively in the absence of an inducer where the regulatory regions comprise an inducible promoter.

The chimeric gene is introduced into the appropriate host by cloning it into a suitable expression vector. Vectors or cassettes useful for the transformation of suitable host cells are well known in the art. Typically, the vector or cassette contains sequences directing transcription and translation of the relevant gene, a selectable marker, and sequences allowing autonomous replication or chromosomal integration. Suitable vectors comprise a region 5' of the coding sequence that harbors transcriptional initiation controls and a region 3' of the DNA fragment which controls transcriptional termination. It is most preferred when both control regions are derived from genes homologous to the host cell, although such control regions need not be derived from the genes native to the specific species chosen as a production host.

Initiation control regions or promoters, which are useful to drive expression of the instant ORF in the desired host cell are numerous and familiar to those skilled in the art, including but not limited to CYC1, HIS3, GAL1, GAL10, ADH1, PGK, PHO5, GAPDH, ADC1, TRP1, URA3, LEU2, ENO, TPI (useful for expression in *Saccharomyces*); AOX1 (useful for expression in *Pichia*); and lac, trp, lP_L , lP_R , T7, tac, P_{BAD} , and trc (useful for expression in *Escherichia coli*). Examples include at least one of the promoters selected from the groups consisting of the tryptophan operon promoter P_{trp} of *E. coli*, a lactose operon promoter $Plac$ of *E. coli*, a P_{tac} promoter of *E. coli*, a phage lambda right promoter P_R , a phage lambda left promoter P_L , a T7 promoter, and a promoter of the GAP gene from *Pichia pastoris*, or is at least one strong promoter selected from the group of microorganisms consisting of *Comamonas*, *Corynebacterium*, *Brevibacterium*, *Rhodococcus*, *Azotobacter*, *Citrobacter*, *Enterobacter*, *Clostridium*, *Klebsiella*, *Salmonella*, *Lactobacillus*, *Aspergillus*, *Saccharomyces*, *Pichia*, *Zygosaccharomyces*, *Kluyveromyces*, *Candida*, *Hansenula*, *Dunaliella*, *Debaryomyces*, *Mucor*, *Torulopsis*, *Methylobacteria*, *Bacillus*, *Escherichia*, *Pseudomonas*, *Rhizobium*, and *Streptomyces*.

Termination control regions may also be derived from various genes native to the preferred hosts. Optionally, a termination site may be unnecessary; however, it is most preferred if included.

Additionally, the inserted genetic material may include a ribosome binding site. The ribosome binding site may be from a phage lambda CII gene or is selected from the group consisting of ribosome binding sites from a gene of *Comamonas*, *Corynebacterium*, *Brevibacterium*, *Rhodococcus*, *Azotobacter*, *Citrobacter*, *Enterobacter*, *Clostridium*, *Klebsiella*, *Salmonella*, *Lactobacillus*,

Aspergillus, Saccharomyces, Zygosaccharomyces, Pichia, Kluyveromyces, Candida, Hansenula, Dunaliella, Debaryomyces, Mucor, Torulopsis, Methylobacteria, Bacillus, Escherichia, Pseudomonas, Rhizobium, and Streptomyces.

5 Optionally, the instant gene product may preferably be a secretion product of the transformed host. Secretion of desired proteins into the growth media simplifies purification procedures and reduces costs. Secretion signal sequences are often useful in facilitating the active transport of expressible proteins across cell membranes. A transformed host capable of secretion may be created by
10 incorporating in the host a DNA sequence that codes for a secretion signal. Methods for choosing appropriate signal sequences are well known in the art (see for example EP 546049; WO 9324631). The secretion signal DNA may be located between the expression-controlling DNA and the instant coding sequence or coding sequence fragment, and in reading frame with the latter.

15 Fermentations, which may be run in the batch, fed-batch, or continuous mode, are common and well known in the art (Thomas D. Brock in *Biotechnology: A Textbook of Industrial Microbiology*, Second Edition (1989) Sinauer Associates, Inc.; Sunderland et al., *Appl. Biochem. Biotechnol.* (1992) 36:227).

20 The present nucleotide fragments may be used to produce gene products having enhanced or altered activity levels. Specifically, the nucleotide fragments can be used to produce a mutated microbial gene encoding a protein characterized by an increased nitrilase activity on nitrile-containing substrates and or an increased enzyme stability, one or both characteristics increased relative to the
25 nitrilase activity of the native microbial gene. Various methods are known for mutating a native gene sequence to produce a gene product with altered or increased activity relative to the native gene sequence. These include, but are not limited to, directed evolution, random mutagenesis, domain swapping (using zinc finger domains, or restriction enzymes), rational design, error prone PCR
30 (Melnikov et al., *Nucleic Acids Research* (1999) 27(4):1056-1062); site-directed mutagenesis (Coombs et al., in *Proteins* (1998) 259-311, R. H. Angeletti (Ed.), Academic Press, San Diego, CA), and "gene shuffling" (US 5,605,793; US 5,811,238; US 5,830,721; and US 5,837,458, incorporated herein by reference).

35 Definitions Of Abbreviations And Terms:

 In this specification, a number of terms and abbreviations are used in the manner defined below.

The abbreviations in the specification correspond to units of measure, techniques, properties, or compounds as follows: "sec" means second(s), "min" means minute(s), "h" means hour(s), "d" means day(s), "μL" means microliter, "mL" means milliliters, "L" means liters, "mM" means millimolar, "M" means molar, "mmol" means millimole(s), "Amp^r" means ampicillin resistance, "Amp^s" means ampicillin sensitivity, "kb" means kilo base, "kd" means kilodaltons, "nm" means nanometers, and "wt" means weight. "ORF" means open reading frame, "PCR" means polymerase chain reaction, "SSC" means saline-sodium citrate buffer, "HPLC" means high performance liquid chromatography, "ca" means approximately, "dcw" means dry cell weight, "O.D." means optical density at the designated wavelength, "IU" means International Units, "MGN" means 2-methylglutaronitrile, "4-CPA" means 4-cyanopentanoic acid, and "IPTG" means isopropyl β-D-thiogalactopyranoside.

"Enzyme catalyst" refers to a catalyst characterized by a specific nitrilase activity on nitrile-containing substrates.

"Hydrogenation catalyst" refers to a material that accelerates hydrogenation without itself being consumed or undergoing a chemical change. Hydrogenation catalysts suitable for use in this invention include, but are not limited to, the various platinum metals, such as iridium, osmium, rhodium, ruthenium, platinum, and palladium; also various other transition metals such as cobalt, copper, nickel and zinc. The catalyst may be unsupported, (for example, as Raney nickel or platinum oxide), or it may be supported (for example, as palladium on carbon, platinum on alumina, or nickel on kieselguhr).

The terms "host cell" and "host organism" refer to a cell capable of receiving foreign or heterologous genes, gene fragments, or DNA fragments.

The term "mesophilic bacterium" refers to a bacterium living in the temperature range near that of warm-blooded animals, and usually showing a growth temperature optimum between 25 and 40 °C.

The terms "recombinant organism", "transformed host", "transformant", "transgenic organism", and "transformed microbial host" refer to a host organism having been transformed with heterologous or foreign DNA. The recombinant organisms of the present invention express foreign coding sequences or genes which encode active nitrilase enzyme. "Transformation" refers to the transfer of a DNA fragment into the genome of a host organism, resulting in genetically stable inheritance. "Transformation cassette" refers to a specific fragment of DNA containing a set of genetic elements conveniently arranged for insertion into a host cell, usually as part of a plasmid. "Expression cassette" refers to a specific fragment of DNA containing a set of genetic elements conveniently arranged for

insertion into a host cell, usually as part of a plasmid, that also allows for enhanced gene expression in the host.

5 The terms “plasmid” and “vector” refer to an extra chromosomal element often carrying genes which are not part of the central metabolism of the host cell, and usually in the form of circular double-stranded DNA molecules. Such elements may be autonomously replicating sequences, genome integrating sequences, phage or nucleotide sequences, linear or circular, of a single- or double-stranded DNA or RNA, derived from any source, in which a number of nucleotide sequences have been joined or recombined into a unique construction.

10 The term “nucleic acid” refers to complex compounds of high molecular weight occurring in living cells, the fundamental units of which are nucleotides linked together with phosphate bridges. Nucleic acids are subdivided into two types: ribonucleic acid (RNA) and deoxyribonucleic acid (DNA).

15 The letters “A”, “G”, “T”, and “C” when referred to in the context of nucleic acids, mean the purine bases (Adenine ($C_5H_5N_5$) and Guanine ($C_5H_5N_5O$)) and the pyrimidine bases (Thymine ($C_5H_6N_2O_2$) and Cytosine ($C_4H_5N_3O$)), respectively.

20 The term “complementary” is used to describe the relationship between nucleotide bases that are hybridizable to one another. For example, with respect to DNA, adenosine is complementary to thymine and cytosine is complementary to guanine. cDNA is a single stranded DNA complementary to an RNA synthesized from it by reverse transcription *in vitro*. Anti-sense RNA is an RNA molecule complementary to another RNA.

25 The terms “coding sequence” or “coding region” refers to a DNA sequence that codes for a specific amino acid sequence. The terms “ORF” and “open reading frame” and “coding sequence” and “coding region” are used interchangeably to refer to a portion of DNA sequence that translates into a protein. ORFs are usually delineated in the sequence by three base pairs designating the start (a start codon) and three base pairs designating the stop (a stop codon) in the translation of the DNA sequence into the protein sequence.

30 The terms “nucleic acid fragment” or “nucleotide fragment” refer to a fragment of DNA that may encode a gene and/or regulatory sequences preceding (5', upstream) or following (3', downstream) the coding sequence. A “fragment” constitutes a fraction of the complete nucleic acid sequence of a particular region.

35 A fragment may constitute an entire gene.

The terms “restriction endonuclease” and “restriction enzyme” refer to an enzyme which catalyzes hydrolytic cleavage within a specific nucleotide sequence in double-stranded DNA.

The term "oligonucleotide" refers to primers, probes, oligomer fragments to be detected, labeled-replication blocking probes, and oligomer controls, and refers generically to polydeoxyribonucleotides (containing 2-deoxy-D-ribose), to polyribonucleotides (containing D-ribose) and to any polynucleotide which is an N-glycoside of a purine or pyrimidine base (nucleotide), or modified purine or pyrimidine base. Also included in the definition of "oligonucleotide" are nucleic acid analogs (e.g., peptide nucleic acids) and those that have been structurally modified (e.g., phosphorothiolate linkages) (See also Thuong et al., *Biochimie* (1985) Jul-Aug 67(7-8):673-684.) There is no intended distinction between the length of a "nucleic acid", "polynucleotide" or an "oligonucleotide".

The term "primer" refers to an oligonucleotide (synthetic or occurring naturally), which acts as a point of initiation of nucleic acid synthesis or replication along a complementary strand when placed under conditions in which synthesis of a complementary strand is catalyzed by a polymerase.

The term "probe" refers to an oligonucleotide (synthetic or occurring naturally), that is significantly complementary to a "fragment" and forms a duplexed structure by hybridization with at least one strand of the fragment.

"Suitable regulatory sequences" refer to nucleotide sequences which influence the transcription, RNA processing, RNA stability, or translation of the associated coding sequence and which are located upstream (5' noncoding sequences), within, or downstream (3' noncoding sequences) of a coding sequence. Regulatory sequences may include promoters, translation leader sequences, introns, and polyadenylation recognition sequences.

"Promoter" refers to a DNA sequence capable of controlling the expression of a coding sequence or functional RNA. In general, a coding sequence is located 3' to a promoter sequence. Promoters may be derived in their entirety from a native gene, or be composed of different elements derived from different promoters found in nature, or even comprise synthetic DNA segments. It is understood by those skilled in the art that different promoters may direct the expression of a gene in different tissues or cell types, or at different stages of development, or in response to different environmental conditions. Promoters that cause a gene to be expressed in most cell types at most times or under most environmental conditions are commonly referred to as "constitutive promoters". Promoters that cause a gene to be expressed only in the presence of a particular compound or environmental condition are commonly referred to as "inducible promoters". Since in most cases the exact boundaries of regulatory sequences have not been completely defined, DNA fragments of different lengths may have identical promoter activity.

The term “operably linked” refers to the association of nucleic acid sequences on a single nucleic acid fragment so that the function of one is affected by the other. For example, a promoter is operably linked with a coding sequence when it is capable of affecting the expression of that coding sequence (i.e., that the coding sequence is under the transcriptional control of the promoter). Coding sequences can be operably linked to regulatory sequences in sense or antisense orientation.

The “3’ non-coding sequences” refer to DNA sequences located downstream of a coding sequence and include polyadenylation recognition sequences and other sequences encoding regulatory signals capable of affecting mRNA processing or gene expression. The polyadenylation signal is usually characterized by affecting the addition of polyadenylic acid tracts to the 3’ end of the mRNA precursor.

“Gene” refers to a nucleic acid fragment that expresses a specific protein, including regulatory sequences preceding (5’ non-coding sequences) and following (3’ non-coding sequences) the coding sequence. “Native gene” refers to a gene with its own regulatory sequences in an arrangement as found in nature. “Chimeric gene” refers to any gene that is not a native gene, comprising regulatory and coding sequences that are not found together in nature. Accordingly, a chimeric gene may comprise regulatory sequences and coding sequences that are derived from different sources, or regulatory sequences and coding sequences derived from the same source, but arranged in a manner different than that found in nature. “Endogenous gene” refers to a native gene in its natural location in the genome of the native organism. A “foreign” gene refers to a gene not normally found in the host organism, but that is introduced into the host organism by gene transfer. Foreign genes can comprise native genes inserted into a non-native organism, or chimeric genes. A “transgene” is a gene that has been introduced into the genome by a transformation procedure.

“Synthetic genes” can be assembled from oligonucleotide building blocks that are chemically synthesized using procedures known to those skilled in the art. These building blocks are ligated and annealed to form gene segments that are then enzymatically assembled to construct the entire gene. “Chemically synthesized”, as related to a sequence of DNA, means that the component nucleotides were assembled *in vitro*. Manual chemical synthesis of DNA may be accomplished using well-established procedures, or automated chemical synthesis can be performed using one of a number of commercially available machines.

The skilled artisan is well aware of the “codon-bias” exhibited by a specific host cell in using nucleotide codons to specify a given amino acid.

Therefore, when synthesizing a gene for improved expression in a host cell, it is desirable to design the gene such that its codon usage reflects the preferred codon bias of the host cell. A survey of genes derived from the host cell where sequence information is available can determine its codon bias.

- 5 The term “expression” means the transcription and translation to gene product from a gene coding for the sequence of the gene product, usually a protein.

 The terms “protein”, “polypeptide”, and “peptide” are used interchangeably to refer to the gene product expressed. “Mature” protein refers to
10 a post-translationally processed polypeptide (i.e., one from which any pre- or pro-peptides present in the primary translation product have been removed). “Precursor” protein refers to the primary product of translation of mRNA (i.e., with pre- and pro-peptides still present). Pre- and pro-peptides may be, but are not limited to, intracellular localization signals.

- 15 The term “percent identity” is a relationship between two or more polypeptide sequences or two or more polynucleotide sequences, as determined by comparing the sequences. “Identity” also means the degree of sequence relatedness between polypeptide or polynucleotide sequences, as the case may be, as determined by the match between strings of such sequences.

- 20 “Similarity” is a descriptive term that only implies that two sequences, by some criterion, resemble each other and carries no suggestion as to their origins or ancestor. “Homology” refers specifically to similarity due to descent from a common ancestor. On the basis of similarity relationships among a group of sequences, it may be possible to infer homology, but outside of an explicit
25 laboratory model system, descent from a common ancestor remains hypothetical. (States et al., Similarity and Homogeneity (1992) pp. 89-92, in J. Sequence Analysis Primer, Gribskov, M. and Devereux, J., (Eds.), Freeman and Co.).

- “Identity” and “similarity” can be readily calculated by known methods including, but not limited to, those described in Computational Molecular Biology
30 (Lesk, A. M., ed.) Oxford University Press, New York (1988); Biocomputing: Informatics and Genome Projects (Smith, D. W., ed.) Academic Press, New York (1993); Computer Analysis of Sequence Data, Part I (Griffin, A. M., and Griffin, H. G., eds.) Humana Press, Totowa, New Jersey (1994); Sequence Analysis in Molecular Biology (von Heinje, G., ed.) Academic Press (1987); and Sequence
35 Analysis Primer (Gribskov, M. and Devereux, J., Eds.) Stockton Press, New York (1991). Preferred methods to determine identity are designed to give the best match between the sequences tested.

Preferred computer program methods to determine the identity and similarity between two sequences include, but are not limited to, the Wisconsin Package Version 9.0 and 10.0 Genetics Computer Group (GCG) Gap program found in the GCG program package, BLASTP, BLASTN, and FASTA (Pearson et al., *Proc. Natl. Acad. Sci. USA.* (1988) 85:2444-2448). The BLAST X program is publicly available from NCBI and other sources (BLAST Manual, Altschul et al., *Natl. Cent. Biotechnol. Inf.*, Natl. Library Med. (NCBI NLM) NIH, Bethesda, MD 20894; Altschul et al., *J. Mol. Biol.* (1990) 215:403-410).

The term "substantially similar" refers to nucleic acid fragments wherein changes in one or more nucleotide bases results in substitution of one or more amino acids, but do not affect the functional properties of the protein encoded by the DNA sequence. "Substantially similar" also refers to nucleic acid fragments wherein changes in one or more nucleotide bases does not affect the ability of the nucleic acid fragment to mediate alteration of gene expression by antisense or co-suppression technology. "Substantially similar" also refers to modifications of the nucleic acid fragments of the instant invention such as substitution, deletion or insertion of one or more nucleotide bases that do not substantially affect the functional properties of the resulting transcript.

"Codon degeneracy" refers to the divergence in the genetic code permitting variation of the nucleotide sequence without affecting the amino acid sequence of an encoded polypeptide. For example, it is well known in the art that the triplet codons CTT, CTC, CTA, and CTG all code for the amino acid leucine (Atlas, R., *Principles of Microbiology*). It is also well known in the art that alterations in a gene that produce a chemically-equivalent amino acid at a given site, but do not affect the functional properties of the encoded protein, are common. Thus, a codon for the amino acid alanine, a hydrophobic amino acid, may be substituted by a codon encoding another less hydrophobic residue (such as glycine) or a more hydrophobic residue (such as valine, leucine, or isoleucine) without affecting the functional properties of the encoded protein. Similarly, substitution of one negatively charged residue for another (such as aspartic acid for glutamic acid) or one positively charged residue for another (such as lysine for arginine) can also be expected to produce a functionally equivalent product. Nucleotide changes that result in alteration of the N-terminal and C-terminal portions of the protein molecule would also not be expected to alter the activity of the protein. Each of the proposed modifications is well within the routine skill in the art, as is determining if the biological activity of the encoded products is retained.

Moreover, the skilled artisan recognizes that substantially similar nucleotide sequences encompassed by this invention are also defined by their

ability to hybridize, under stringent conditions, with the sequences exemplified herein.

Typically, stringent conditions are those in which the salt concentration is less than about 1.5 M Na ion (typically about 0.01 to 1.0 M Na ion concentration or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30 °C for short probes (e.g., 10 to 50 nucleotides) and at least about 60 °C for long probes (e.g., greater than 50 nucleotides). Stringent conditions may also be achieved by adding destabilizing agents such as formamide. Exemplary low stringency conditions include hybridization with a buffer solution of 6X SSC (1 M NaCl), 30 to 35 % formamide, 1 % SDS (sodium dodecyl sulphate) at 37 °C, and a wash in 1X to 2X SSC (20X SSC = 3.0 M NaCl/0.3 M trisodium citrate) at 50 to 55 °C. Exemplary moderate stringency conditions include hybridization in 6X SSC (1 M NaCl), 40 to 45 % formamide, 1 % SDS at 37 °C, and a wash in 0.5X to 1X SSC at 55 to 60 °C. Exemplary high stringency conditions include hybridization in 6X SSC (1 M NaCl), 50 % formamide, 1 % SDS at 37 °C, and a wash in 0.1X SSC at 60 to 65 °C.

“Specificity” is typically a function of post-hybridization washes, the critical factors being the ionic strength and temperature of the final wash solution. The melting temperature T_m of a probe–target hybrid can be calculated to provide a starting point for determining correct stringency conditions. The T_m is the temperature (under defined ionic strength and pH) at which 50 % of a complementary target sequence hybridizes to a perfectly matched probe. For DNA-DNA hybrids, the T_m can be approximated from the equation of Meinkoth and Wahl (*Anal. Biochem.* (1984) 138:267-284) as follows:

$$T_m = 81.5\text{ }^{\circ}\text{C} + 16.6 (\log M) + 0.41 (\% \text{ G+C}) - 0.61 (\% \text{ form}) - 500/L;$$
where M is the molarity of monovalent cations, % G+C is the percentage of guanosine and cytosine nucleotides in the DNA, % form is the percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs.

T_m is reduced by about 1°C for each 1 % of mismatching; thus, T_m , hybridization and/or wash conditions can be adjusted to hybridize to sequences of the desired identity. For example, if sequences with >90 % identity are sought, the T_m can be decreased 10°C. Generally, stringent conditions are selected to be about 5 °C lower than the thermal melting point T_m for the specific sequence and its complement at a defined ionic strength and pH. However, severely stringent conditions can use a hybridization and/or wash at

1, 2, 3, or 4 °C lower than the thermal melting point T_m ; moderately stringent conditions can use a hybridization and/or wash at 6, 7, 8, 9, or 10 °C lower than the thermal melting point T_m ; and low stringency conditions can use a hybridization and/or wash at 11, 12, 13, 14, 15, or 20 °C lower than the thermal melting point T_m . Using the equation, hybridization and wash compositions, and desired T_m , those of ordinary skill will understand that variations in the stringency of hybridization and/or wash solutions are inherently described. If the desired degree of mismatching results in a T_m of less than 45 °C (aqueous solution) or 32 °C (formamide solution) it is preferred to increase the SSC concentration so that a higher temperature can be used.

Hybridization requires that the two nucleic acids contain complementary sequences, although depending on the stringency of the hybridization, mismatches between bases are possible. The appropriate stringency for hybridizing nucleic acids depends on the length of the nucleic acids and the degree of complementation, variables well known in the art. The greater the degree of similarity or homology between two nucleotide sequences, the greater the value of T_m for hybrids of nucleic acids having those sequences. The relative stability (corresponding to higher T_m) of nucleic acid hybridizations decreases in the following order: RNA:RNA, DNA:RNA, DNA:DNA. For hybrids of greater than 100 nucleotides in length, equations for calculating T_m have been derived (see Sambrook et al., *supra*, 9.50-9.51). For hybridizations with shorter nucleic acids, i.e., oligonucleotides, the position of mismatches becomes more important, and the length of the oligonucleotide determines its specificity (see Sambrook et al., *supra*, 11.7-11.8). In one embodiment the length for a hybridizable nucleic acid is at least about 10 nucleotides. Preferable a minimum length for a hybridizable nucleic acid is at least about 15 nucleotides; more preferably at least about 20 nucleotides; and most preferably the length is at least 30 nucleotides. Furthermore, the skilled artisan will recognize that the temperature and wash solution salt concentration may be adjusted as necessary according to factors such as length of the probe and G+C composition of the target DNA.

An extensive guide to the hybridization of nucleic acids is found in Tijssen, *Laboratory Techniques in Biochemistry and Molecular Biology-Hybridization with Nucleic Acid Probes*, Part I, Chapter 2 "Overview of principles of hybridization and the strategy of nucleic acid probe assays" (1993) Elsevier, New York, and in *Current Protocols in Molecular Biology*,

(1995) Chapter 2, Ausubel et al. (Eds.), Greene Publishing and Wiley-Interscience, New York.

GENERAL METHODS

5 All HPLC methods were performed according to Gavagan et al., *J. Org. Chem.* (1998) 63:4792-4801.

10 For computational nucleic acid and protein sequence assembly and analyses (including determination of identity and similarity), Applicants used the Wisconsin Package Version 9.0 and 10.0 Genetics Computer Group (GCG) Gap program found in the GCG program package (using the Needleman and Wunsch algorithm with their standard default values of gap creation penalty=50 and gap extension penalty=3 (Devereux et al., *Nucleic Acids Res.* (1984) 12:387-395)), Sequencher Version and Vector NTI Deluxe v 4.0.3 software and database packages. Unless specified otherwise, all default parameters were used.

15 Materials and methods suitable for the maintenance and growth of bacterial cultures are well known in the art. Techniques suitable for use in the following examples may be found as set out in Manual of Methods for General Bacteriology (1994) (Phillipp Gerhardt et al. (Eds.), American Society for Microbiology, Washington, DC.) or as set out in Biotechnology: A Textbook of Industrial Microbiology (1989) Second Edition, (Thomas D. Brock, Sinauer Associates, Inc., Sunderland, MA).

Nitrile-containing substrates suitable for use in this invention are dinitriles having the formula NC-R-CN where R is an alkylene group having from about 1 to about 10 carbons. A more preferred nitrile-containing substrate is an aliphatic α,ω -dinitrile having the formula

25
$$\text{NCCX}_a(\text{R})(\text{CH}_2)_n\text{CN},$$

where $a = 0$ or 1 , where $X = \text{hydrogen}$ when $a = 1$, and $R = \text{H}$, alkyl or substituted alkyl, or alkenyl or substituted alkenyl, or alkylidene or substituted alkylidene, and where $n = 1$ or 2 . Most preferred for use as a nitrile-containing substrate in this invention is 2-methylglutaronitrile.

30 In an improved method for producing five-membered or six-membered ring lactams with biocatalysts comprising the biological units claimed herein, preferred is an aliphatic α,ω -dinitrile of either the formula:

N-terminal amino acid sequence and sequences of peptide digestion products from the purified *Acidovorax facilis* 72W nitrilase protein were determined. Nitrilase purified by gel filtration was sequenced following trypsin digestion using methods known to the art, including cysteine modification by reduction followed by alkylation with 4-vinylpyridine (See, for example, Matsudaira, *Methods Enzymol.* (1990) 182 (Guide Protein Purif.):602-13 or Allen, Sequencing of Proteins and Peptides. In: Laboratory Techniques in Biochemistry and Molecular Biology (Burdon, R. H. and van Knippenberg, P. H., Eds), Elsevier, Amsterdam, New York, Oxford (1989)).

Database searches were run on GCG using Wisconsin Software Package 9.1 (Genetics Computer Group, Madison, WI). Determined amino acid sequences were compared to protein sequence information contained in the SWISS-PROT database. Ten oligopeptides isolated from the digest of the purified *Acidovorax facilis* 72W nitrilase protein had amino acid sequences with >60 % similarity to other known nitrilase proteins. This indicates that the purified protein has a high probability of containing a nitrilase enzyme.

B. Isolation of a DNA Fragment Containing the Nitrilase Gene and Expression in *E. coli*:

To identify possible DNA sequences with homology to known nitrilase genes, degenerate oligonucleotide primers were designed and synthesized for use as PCR primers. The design of these PCR primers was based on conserved coding regions found in bacterial nitrilase sequences available from the GenBank database (GenBank Accession Numbers D12583, J03196, D13419, L32589, D67026). Genomic DNA was isolated from *Acidovorax facilis* 72W (ATCC 55746) by standard methods (Maniatis) modified by adding two rounds of phenol-chloroform extraction. DNA so isolated was used as a target for PCR with numerous degenerate primer combinations. The resulting amplified products were cloned into the pGem-T vector and sequenced by methods common to the art. From experiments with ten different PCR primer pairs, only one product found in plasmid pJJ 28-5, resulting from primers 1F (SEQ ID NO:1) and 7R (SEQ ID NO:2), yielded a 385 bp DNA fragment (SEQ ID NO:3) with 74.1 % identity to a region of the nitrilase DNA sequence from *Rhodococcus rhodochrous* K22 (GenBank Accession Number D12583). In addition, the putative protein product from this 385 bp DNA fragment contained a number of peptide sequences matching those isolated from the purified 72W nitrilase protein digest. This was additional evidence that the identified 385 bp fragment contained a portion of the desired 72W nitrilase gene.

With the discovery of this partial gene sequence with high homology to other known nitrilase genes, Applicants have discovered a piece of DNA useful as a probe to identify recombinant clones containing the 72W nitrilase gene in any DNA library. In addition, the successful use of SEQ ID NO:1 and SEQ ID NO:2 as degenerate PCR primers to discover a novel nitrilase gene shows that

5 Applicants have discovered DNA sequences that have general utility as a means of identifying and isolating unknown nitrilases from bacterial strains.

In order to map the complete nitrilase gene to a single restriction fragment, Southern hybridizations were performed on *Acidovorax facilis* 72W genomic

10 DNA. High molecular weight genomic DNA was isolated from *Acidovorax facilis* 72W cells by using Qiagen genomic tip-100/G DNA isolation kit (Qiagen Inc., USA). 1 µg genomic DNA was digested with a variety of restriction enzymes, resolved on 1 % agarose gel followed by transfer to nylon membrane. Restriction fragments immobilized on nylon membrane were probed with 385 bp

15 partial nitrilase fragment (SEQ ID NO:3) by Southern hybridizations. The nitrilase gene was mapped to a single restriction fragment when genomic DNA was digested with any of the following restriction enzymes: *Bam*HI, *Bcl*II, *Bgl*III, *Cla*I, *Eag*I, *Kpn*I, *Nar*I, *Nhe*I, *Not*I, *Nsi*II, *Pst*I, *Sal*II, *Spe*I, *Sst*I, *Xba*I, *Xho*I. These observations provided strong evidence for the presence of a single nitrilase gene in

20 *Acidovorax facilis* 72W (ATCC 55746). *Eco*RI digests, from the known partial gene sequence (SEQ ID NO:3) which contains an *Eco*RI site within itself, yielded two bands. Digests with *Pst*I yielded a single, 4.1 kb band that reacted with the probe, suggesting that the nitrilase homolog was present on a 4.1 kb *Pst*I fragment from the chromosome. This information was critical for selecting a restriction

25 enzyme (in this case *Pst*I) to generate an enriched library of genomic fragments to isolate the complete nitrilase gene.

To construct a library, *Acidovorax facilis* 72W genomic DNA was digested with *Pst*I and resolved by gel electrophoresis. Fragments ranging in size from 2.5–7 kilobases were recovered from the gel and ligated into the vector

30 pBluescript II SK(+) (Stratagene, La Jolla, CA, USA). The ligated DNA was electrotransformed into *E. coli* DH10B electromax cells (Life Technologies, Rockville, MD, USA). The resultant library represented 4 x 10⁶ independent, recombinant clones of *Acidovorax facilis* 72W *Pst*I fragments. In order to screen the library, plasmids were prepared from cells scraped from plates.

35 The above plasmid library was transformed into *E. coli* DH5α. A labeled probe fragment containing the 385 bp nitrilase gene fragment from pJJ28-5 was used to screen the library by colony hybridization. Three colonies that showed positive hybridization with the nitrilase probe were grown in liquid cultures in the

presence of ampicillin. Plasmids from these colonies (pnit4, pnit5, and pnit6) showed identical restriction fragment patterns when digested with *Pst*I, *Eco*RI, and *Hind*III, suggesting that these plasmids contained identical inserts. *Pst*I digestion yielded a 4.1 kb insert fragment confirming Southern hybridization results. Inserts in all the above plasmids contained one internal *Eco*RI and one *Hind*III site, respectively, as expected from the partial gene sequence already identified (SEQ ID NO:3).

The inserts in plasmids pnit4, pnit5, and pnit6 had identical nucleotide sequences (SEQ ID NO:4) containing an ORF encoding a 369 amino acid sequence (SEQ ID NO:5) similar in length to other nitrilases. The primary amino acid sequence is 71 % identical to the nitrilase from *Rhodococcus rhodochrous* K22 (GenBank Accession Number D12583). The ORF start codon is GTG, which yields a protein beginning with a valine instead of a more common start of methionine. The amino acid sequence (SEQ ID NO:5) encoded by the above ORF (SEQ ID NO:4) was scanned in the PROSITE database of protein families and domains using Scanprosite ([www. Espay.ch/tools/scnpsit1.html](http://www.Espay.ch/tools/scnpsit1.html)) and Profilescan ([www .isrec.isb-sib.ch/software/PFSCAN-form.html](http://www.isrec.isb-sib.ch/software/PFSCAN-form.html)) and was found to contain signature patterns conserved in all known nitrilases. Based on this data, cysteine at position 164 (SEQ ID NOs:5 and 14) is proposed to be the active site cysteine in *Acidovorax facilis* 72W nitrilase. The sequence similarity to known nitrilases as well as the presence of the nitrilase signature together provided evidence that the gene for nitrilase was present on the 4.1 kb *Pst*I fragment of pnit4, pnit5, and pnit6 on an ORF (SEQ ID NO:4) encoding a 369 amino acids sequence (SEQ ID NO:5).

The instant invention thus provides a plasmid clone DH5 α :pnit4 containing the complete *Acidovorax facilis* 72W nitrilase gene and the flanking chromosomal DNA in a form convenient for manipulation.

The *Acidovorax facilis* 72W nitrilase ORF identified in the 4.1 kb *Pst*I fragment from pnit4 was cloned into a number of pet expression vectors based on T7 promoter-T7 RNA polymerase system for overexpression (Studier et al., *Meth. Enzymol.* (1990) 185:60-89). More specifically, the ORF was cloned into pET-3c and pET-21a (both, Novagen, Madison, WI). The pET-3c nitrilase construct (pnitex2 (Example 6)) and pET-21a construct (pSW91(Example 7)) both have the start codon (GTG) of native nitrilase ORF replaced with ATG. An additional pET-21a construct (pSW90 (Example 7)) yields a modified form that contains an 11 amino acid T7 tag fusion at the N-terminus of the protein. The plasmid pnitex2 was transformed into two different host strains BL21(DE3) and BL21-SI yielding strains SS1001 and SS1002 respectively (Example 6). These strains allowed

expression of nitrilase with or without induction by IPTG (strain SS1001, Example 6) or induction by NaCl (strain SS1002, Example 6). The plasmids pSW90 and pSW91 were transformed into BL21(DE3) yielding strains SW90 and SW91 respectively (Example 7) both of which expressed enzymatically active nitrilase with or without induction by IPTG. When the above *E. coli* strains containing these constructs were induced according to protocols provided by the manufacturer, SS1001, SW90, and SW91 produced a specific protein of the expected molecular weight, approximately 40 kd. In addition, when tested for nitrilase enzyme activity (indicated by the ability to catalyze the conversion of MGN to 4-CPA), all expression systems (SS1001, SS1002, SW90, and SW91) catalyzed this reaction, showing that the genetically engineered *E. coli* strains were able to produce active *Acidovorax facilis* 72W nitrilase enzyme (Examples 6 and 7).

C. Production of *Acidovorax facilis* 72W (ATCC 55746) and *E. coli* expression strains

Growth of *Acidovorax facilis* strain 72W (ATCC 55746)

One frozen seed lot vial was thawed and the 1 mL contents placed in 500 mL of sterile Inoculum Medium listed below. The inoculum was grown at 30 °C with shaking at 250 rpm in a 2 L flask for 24-30 h.

Inoculum Medium

<u>Component:</u>	<u>Final Concentration:</u>
Potassium phosphate, monobasic	1.5 g/L
Potassium phosphate, dibasic	3.4 g/L
Ammonium sulfate	1.5 g/L
Trisodium citrate, dihydrate	1 g/L
Magnesium sulfate, heptahydrate	0.4 g/L
Trace metal solution (below)	1 mL /L
Amberex 695 (Universal Foods)	1 g/L
Glycerol (sterilized separately)	8 g/L

Trace Metal Solution

<u>Component:</u>	<u>Stock Concentration:</u>
Hydrochloric Acid	10 mL/L
Calcium chloride, dihydrate	11.4 g/L
Manganese Sulfate, monohydrate	1.23 g/L
Copper sulfate, pentahydrate	0.63 g/L
Cobalt chloride, hexahydrate	0.16 g/L
Boric Acid	0.91 g/L

	Zinc sulfate, heptahydrate	1.77 g/L
	Sodium molybdate, dihydrate	0.05 g/L
	Vanadyl sulfate, dihydrate	0.08 g/L
	Nickel nitrate, hexahydrate	0.04 g/L
5	Sodium selenite	0.04 g/L
	Ferrous sulfate, heptahydrate	6.0 g/L

10 The inoculum from the shake flask was transferred aseptically to a presterilized Braun Biostat C fermenter containing the Fermenter Medium listed below.

Fermenter Medium

	<u>Component:</u>	<u>Final Concentration:</u>
	Potassium phosphate, monobasic	0.39 g/L
	Potassium phosphate, dibasic	0.39 g/L
15	Difco yeast extract	5.0 g/L

20 Growth occurred under the following conditions: 32 °C, pH 6.8-7.0, dissolved oxygen at 25 % of saturation. At inoculation, the fermenter contained 8.5 L of Fermenter Medium plus 218 g of Nutrient Feed solution, giving a starting concentration of approximately 7 g/L glycerol. The Nutrient Feed solution included the following components which were sterilized separately and combined after cooling: potassium phosphate, monobasic, 19.6 g in 0.25 L deionized water; magnesium sulfate, heptahydrate, 3.3 g, plus sulfuric acid, 4 mL, in 0.15 L deionized water; Trace Metal solution, 67 mL, plus 400 g glycerol in 0.80 L deionized water. At 18 h post inoculation, feeding of Nutrient Feed solution began. Initially, the Nutrient Feed solution was added at a rate of 0.4 g feed/minute (0.15 g glycerol/min). The culture optical density measured at 550 nm (O.D. 550) was approximately 8-9. At 26 h, the O.D. 550 was 16-18 and the feed rate was increased to 0.9 g feed/min (0.3 g glycerol/min). A final increase in feed rate to 1.8 g feed/min (0.6 g glycerol/min) was made at 34 h. This rate continued to the end of the run (about 42 h). The final O.D. 550 was approximately 65-75 and equivalent to a cell density of 25-30 g dcw/L.

35 Cells were recovered by centrifugation and stored frozen until use. For use as a biocatalyst, cells were heated to 50 °C for 1 h in 0.35 M phosphate buffer (pH 7.3) and then used to catalyze the transformation of MGN into 4-CPA.

Growth of *E. coli* cells for whole cell activity and immobilization:

25 mL of overnight culture of *E. coli* strain SS1001 grown from a single colony was inoculated into 250 mL fresh LB medium. The cultures were grown to mid-log phase and harvested by centrifugation with or without induction with

1 mM IPTG for 1 h. The strain SS1002 was grown in LBON (LB without NaCl) medium and induced with 0.2 M NaCl for 1 h at mid-log phase according to manufacturer's instructions for BL21-SI overexpression strain (Life Technologies, Rockville, MD, USA). The bacterial cell pellets were stored on ice overnight
5 prior to measurement of nitrilase activity. For immobilization, *E. coli* strain SS1001 was grown in a 10 L batch fermentation in LB medium to an O.D. at 600 nm of 8.6. Cells were harvested by centrifugation and stored on wet ice for whole cell activities and immobilization.

Comparing the weight-specific activity of biocatalyst produced from the
10 native strain, as described above, to the genetically engineered biocatalysts from Examples 6, 7, and 15 it is clear that the expression strains SS1001, SS1011, and SW91 produced biocatalysts of substantially greater specific activity (Table 2 in Example 6, Tables 3 and 4 in Example 7, and Table 5 in Example 15) than that of the native strain (Tables 2, 3, 4, and 5).

15 EXAMPLE 1

Purification of Nitrilase Protein

All steps in this procedure were performed at 5 °C and at pH 7.5 unless otherwise stated.

A 25 wt % suspension of *Acidovorax facilis* 72W (ATCC 55746) wet cell
20 paste was prepared in 20 mM Tris, pH 7.5, 0.1 mM phenyl methyl sulfonyl fluoride (PMSF), and 2.0 mM dithiothreitol.

An extract of this suspension was prepared by passage through a French
press (American Instrument Co., Silver Springs, MD, USA) according to methods known to the art. Following a centrifugation at 27,500 g for 30 min to remove cell
25 debris, a 20-55 % ammonium sulfate fractionation of the extract was prepared and then concentrated by overnight precipitation following the addition of solid ammonium sulfate to 65 % of saturation. The concentrated protein precipitate was reconstituted using a minimum volume of 20 mM Tris, pH 7.5 (Buffer A) and desalted over a PD10 column containing Sephadex G-25 resin (Pharmacia).

30 Following desalting, the concentrated protein extract was fractionated by anion exchange chromatography using a column containing 50 mL of Q-Sepharose fast flow (Pharmacia). After loading the column with the concentrated protein extract, the column was washed with three column volumes of Buffer A at a flow rate of 2 mL/min to remove un-adsorbed protein. Adsorbed
35 protein was eluted from the column using a 0-0.5 M NaCl gradient prepared in Buffer A. Elution of protein from the column was monitored at 280 nm. Nitrilase activity was monitored throughout purification using an assay measuring the hydrolysis of benzonitrile to produce benzoic acid. Nitrilase activity eluted at

0.4 M NaCl. Protein components in the 0.4 M NaCl protein fraction were separated by gel electrophoresis (SDS-PAGE) performed under reducing conditions (5 % β -mercaptoethanol) on a 10-15 % SDS polyacrylamide gel. Greater than 50 % of the 0.4 M NaCl protein fraction consisted of a protein with subunit molecular weight of 39.7 kd. This is within the expected molecular weight range for nitrilase enzymes (Cowan et al., *Extremophiles* (1998) 2:207-216). Using methods known to the art, the native molecular weight of the nitrilase was determined to be 570 kd following gel filtration chromatography in 20 mM phosphate buffer at pH 7 using a Hiload 16/60 Superdex 200 column (Pharmacia) which had been calibrated using gel filtration MW standards (Pharmacia #17-0442-01). Following gel filtration, the nitrilase protein was >90 % pure. The specific activity of the purified enzyme was determined to be 35 IU/mg protein using 2-methylglutaronitrile as substrate at 25 °C.

EXAMPLE 2

Isolation of a DNA Fragment Showing High Homology to Known Nitrilases

Genomic DNA was isolated from *Acidovorax facilis* 72W (ATCC 55746) by standard methods (Maniatis) modified by adding two rounds of phenol-chloroform extraction. DNA so isolated was used as a target for amplification by PCR using primers 1F (SEQ ID NO:1) and 7R (SEQ ID NO:2). The resulting amplified products were cloned into the pGem-T vector (Promega, Madison, WI) and sequenced by methods common to the art. Clone pJJ 28-5 yielded a 385 bp DNA fragment (SEQ ID NO:3) with 74.1 % identity to a region of the nitrilase DNA sequence from *Rhodococcus rhodochrous* K 22 (GenBank Accession Number D12583).

EXAMPLE 3

Localization of a Chromosomal Fragment Containing a Sequence with High Homology to the 385 bp Partial-Gene Fragment

1-3 μ g high molecular weight genomic DNA samples from *Acidovorax facilis* 72W were digested with restriction enzyme *Pst*I in a buffer supplied by the manufacturer (Life Technologies (Gibco-BRL), Gaithersburg, MD, USA). Digested samples were resolved on agarose gels and Southern-blotted on nylon membrane. Cutting at restriction sites flanking the insert, a probe containing the 385 bp nitrilase gene fragment (SEQ ID NO:3) was prepared from pJJ28-5. Hybridizations were carried out at 60 °C followed by high stringency washes (0.1 x SSC, 0.1 % SDS, 60 °C for 15 min). Probe labeling, hybridization and detection for dot blots and subsequent Southern blotting experiments, were performed using ECL random prime labeling and detection systems version II, (Amersham International plc, Buckinghamshire, England).

Digests with *Pst*I yielded a single 4.1 kb band upon hybridization with the probe indicating that the nitrilase gene was present on a 4.1 kb *Pst*I fragment of the *Acidovorax facilis* 72W genome.

EXAMPLE 4

5 Construction of *Acidovorax facilis* 72W (ATCC 55746) Genomic Library

5 µg high molecular weight genomic DNA from *Acidovorax facilis* 72W was digested with *Pst*I and resolved on a preparative 1 % agarose gel. Fragments ranging in size from 2.5 to 7 kb were recovered from the gel and ligated into *Pst*I digested and dephosphorylated pBluescript II SK(+) vector (Stratagene, La Jolla, CA, USA). The ligated DNA was electrotransformed into *E. coli* DH10B
10 electromax cells (Life Technologies (Gibco-BRL), Gaithersburg, MD, USA) and transformed cells were plated on LB+ ampicillin (100µg/mL)+ IPTG + X -Gal plates. The resultant library with 95 % recombinant plasmids represented 4 X 10⁶ independent recombinant clones. For library screening and storage, plasmids were
15 prepared from the cells scraped from the plates.

The above plasmid library was transformed into *E. coli* DH5α (Life Technologies, Rockville, MD, USA) cells and the transformants were plated on LB + ampicillin 100 µg/mL plates. After incubating overnight at 37 °C, well-isolated colonies were transferred to nylon membrane and lysed *in situ* to
20 immobilize DNA on membrane. The membranes were probed with labeled nitrilase gene probe described in Example 3. Three colonies that showed positive hybridization with the nitrilase probe were grown in liquid cultures in the presence of ampicillin. Plasmids pnit4, pnit5, and pnit6 yielded identical patterns upon digestion with restriction enzymes *Pst*I, *Eco*RI, and *Hind*III respectively. These
25 results confirmed that identical inserts were present in pnit4, pnit5, and pnit6. Restriction digests yielded a 4.1 kb *Pst*I insert as expected from earlier Southern hybridization.

EXAMPLE 5

Sequence of *Acidovorax facilis* 72W (ATCC 55746) nitrilase ORF

30 The 4.1 kb insert in plasmid pnit4 was sequenced using the standard Sanger dideoxy chain termination method. Sequence analyses using GCG MAP and Vector NTI software revealed the presence of an 1110 bp open reading frame (SEQ ID NO:4; SEQ ID NO:15, bases 332-1441). This ORF was present within a *Bcl*II-*Bgl*III fragment (SEQ ID NO:15, and Figure 1) of the pnit4 insert. A search of
35 GenBank sequence database revealed that this open reading frame (SEQ ID NO:4) had 68 % identity to the nitrilase ORF from *Rhodococcus rhodochrous* K22 (GenBank Accession Number D12583). The 369 amino acids sequence (SEQ ID NO:5) deduced from the open reading frame (SEQ ID NO:4) is 71 % identical to

the *Rhodococcus rhodochrous* K22 nitrilase protein. A scan of the amino acid sequence (SEQ ID NO:5) encoded by *Acidovorax facilis* 72W ORF (SEQ ID NO:4) into the PROSITE database of protein families and domains using Profilescan and Scanprosite tools revealed the presence of signature patterns conserved for all known nitrilases.

EXAMPLE 6

Expression of *Acidovorax facilis* 72W (ATCC 55746)

Nitrilase in *E. coli*

Oligonucleotides SEQ ID NO:6 and SEQ ID NO:7 were used in a PCR reaction to amplify the nitrilase ORF from *Acidovorax facilis* 72W (ATCC 55746) genomic DNA. The PCR product was digested with *NdeI* and *BamHI* and was cloned into *NdeI-BamHI* linearized pET-3c. The start codon of nitrilase ORF in the resultant expression plasmid (pnitex2) is ATG (SEQ ID NO:13) instead of the native GTG codon (SEQ ID NO:4). Accordingly, the amino acid sequence of the nitrilase expressed from pnitex2 has M (methionine) at position 1 (SEQ ID NO:14) instead of the native V (valine) of *Acidovorax facilis* 72W nitrilase (SEQ ID NO:5). Plasmids pnitex2 and pET-3c (control) were transformed into *E. coli* strains BL21(DE3) (Novagen, Madison, WI, USA) to yield SS1001 and BL21(DE3): pET-3c, respectively.

Strains resulting from transforming pnitex2 and pET-3c into BL21-SI (Life Technologies, Rockville, MD) were named SS1002 and BL21-SI:pET-3c, respectively. Plasmid pnitex2 contains nitrilase coding sequence under the control of T7 promoter. Transcription of chromosomally located T7 RNA polymerase, which regulates transcription of a gene under control of the T7 promoter (in this case the chimeric nitrilase gene), is induced by the addition of IPTG in host strain BL21 (DE3) and by the addition of NaCl in host BL21-SI. The above *E. coli* transformants, SS1001 (and the control strain BL21(DE3): pET-3c) and SS1002 (and the control strain BL21-SI: pET-3c), were grown in shake flasks or 10 L fermenters and chimeric nitrilase gene expression was measured with or without induction by adding either IPTG or NaCl following protocols provided by the manufacturers. Crude extracts of strains SS1001, SS1002, and BL21(DE3): pET-3c were prepared and run on SDS-PAGE gels with 5-20 µg total protein per lane. Molecular weight and laser densitometer analysis of the SDS-PAGE gel determined that the strains SS1001 and SS1002 expressed a specific protein band of approximately 40 kd size, corresponding to the expected size of *Acidovorax facilis* 72W nitrilase protein. This band represented > 50 % of total soluble protein in SS1001. The corresponding band was absent in the BL21(DE3): pET-

3c crude extracts. Enzyme assays of the extracts from SS1001 suggested that 12 % of total soluble protein was enzymatically-active nitrilase.

Whole cells of *E. coli* transformants SS1001 and SS1002, along with control strains BL21(DE3): pET-3c and BL21-SI: pET-3c, respectively, were also tested for nitrilase activity. The above transformants were grown to late log phase and the nitrilase activity levels of whole cells with or without induction was compared to the activity of strains harboring the control pET-3c vector only (Table 2). The nitrilase activity was measured by determining the rate of 4-CPA production from MGN. A 50 mg (dry cell weight)/mL cell suspension was prepared in 0.10 M potassium phosphate buffer, pH 7.0. Into a 20-mL glass scintillation vial equipped with a magnetic stir bar was added 3.0 mL of an aqueous solution of 0.40 M MGN at 25 °C. With stirring, 1.0 mL of the cell suspension at 25 °C was added. At 5, 10, and 15 minutes after the addition of the cell suspension, a 180 µL aliquot was removed from the reaction mixture, mixed with 5 µL of 6.0 N HCl and 20 µL of 0.75 M N-methylpropionamide in water (HPLC external standard), centrifuged, and the supernatant analyzed by HPLC for the rate of production of 4-CPA ammonium salt.

A unit of nitrilase activity (IU) is equivalent to production of 1 micromole 4-CPA ammonium salt/min. The activity level is reported as units per gram of dry cell weight and compared to the activity of *A. facilis* 72W.

Table 2: Nitrilase Activity in *E. coli* transformants

Transformant Catalyst	Induction	Nitrilase Activity (IU/g dry cell weight)
<i>E. coli</i> SS1001 (BL21(DE3):pnitex2)	None	360
<i>E. coli</i> SS1001 (BL21(DE3):pnitex2)	IPTG	466
<i>E. coli</i> control (BL21 (DE3):pET-3c)	IPTG	0
<i>E. coli</i> SS1002 (BL21-SI: pnitex2)	NaCl	288
<i>E. coli</i> control (BL21 -SI: pET-3c)	None	0
<i>Acidovorax facilis</i> 72W (ATCC 55746)	Not applicable	271

EXAMPLE 7**Expression of *Acidovorax facilis* 72W nitrilase gene in *E. coli***

- 5 The 72W nitrilase coding sequence was isolated from *Acidovorax facilis* 72W by PCR using primers corresponding to DNA sequence determined at the 5' and 3' ends of the coding sequence. The coding sequence was amplified using primers identified as SEQ ID NO:8 and SEQ ID NO:9 and subcloned into pGEM-T (Promega, Madison, WI). The primer identified as SEQ ID NO:8
- 10 changes the native GTG start codon to ATG. The nitrilase gene fragment was then removed from pGEM-T with *Bam*HI and *Sac*I and subcloned into the *E. coli* expression vector pET-21a (Novagen, Madison, WI), between *Bam*H1 and *Sac*I to generate plasmid pSW90, such that an 11 amino acid T7 tag was encoded at the N-terminus of the nitrilase coding sequence. The coding sequence was also
- 15 amplified using primers identified as SEQ ID NO:10 and SEQ ID NO:9 and subcloned into pGEM-T. The primer identified as SEQ ID NO:10 changes the native GTG start codon to ATG. The gene fragment was then removed from pGEM-T with *Nde*I and subcloned into pET-21a at the *Nde*I site to generate plasmid pSW91, such that no modifications (except for the start codon as
- 20 described) were made to the native nitrilase coding sequence. Plasmids pSW90 and pSW91 were transformed into *E. coli* strain BL21(DE3) (Novagen, Madison, WI), to generate strains SW90 and SW91, respectively. After standard growth and induction (Novagen recommendations) of SW90 and SW91, cell extracts were prepared and examined by SDS-PAGE, and a major protein band of the expected
- 25 size, ~40 kDa, corresponding to 72W nitrilase protein, was observed. In the case

of SW91, the nitrilase protein on the PAGE gel represented > 50 % of total soluble protein. No comparable major band was observed in the control.

E. coli transformant SW91 expressing the *Acidovorax facilis* 72W (ATCC 55746) nitrilase coding sequence was tested for nitrilase activity by measuring the rate of 4-CPA production from MGN as described in Example 6.

Following growth and IPTG induction as recommended by the manufacturer (Novagen, Madison WI), the nitrilase activity level of SW91 was measured and compared to the activity of a control transformant harboring the expression vector without the nitrilase gene, and to typical activity observed for *A. facilis* 72W. The results are shown in Table 3. One unit of nitrilase activity (IU) is equivalent to production of 1 micromole 4-CPA ammonium salt/min, and is reported as units per gram of dry cell weight.

Table 3: Nitrilase activity in SW91 after standard growth and induction

Strain	Nitrilase Activity (IU/g dry cell weight)
<i>E. coli</i> SW91 (BL21(DE3):pSW91)	551
<i>E. coli</i> control (BL21(DE3):pET-21a)	0
<i>Acidovorax facilis</i> 72W (ATCC 55746)	271

E. coli transformant SW91 was also grown at 37 °C with shaking in LB media (Maniatis) without IPTG induction until saturation (12-16 h), after which cells were harvested by centrifugation and assayed for nitrilase activity. The results are shown in Table 4, and compared to typical activity observed for *A. facilis* 72W.

Table 4: Nitrilase activity in SW91 after growth to saturation without induction

Strain	Nitrilase Activity (IU/g dry cell weight)
<i>E. coli</i> SW91 (BL21(DE3):pSW91)	662
<i>Acidovorax facilis</i> 72W (ATCC 55746)	271

EXAMPLE 8

Immobilization of *Acidovorax facilis* 72W or *E. coli*

Transformant SS1001 Cells in Carrageenan

Into a 250 mL media bottle (equipped with magnetic stir bar and
5 containing 64.12 g of distilled, deionized water at 50 °C) was slowly added 3.38 g
of FMC BioPolymer ISAGEL® RG300 carrageenan with rapid stirring. The
mixture was heated to 75-80 °C with rapid stirring until the carrageenan was
completely dissolved, and the resulting solution cooled to 55-56 °C (gelling
temperature ca. 52 °C) in a thermostated water bath. A suspension of either
10 *Acidovorax facilis* 72W cells or *E. coli* transformant SS1001 (12.5 % dry cell
weight) in 0.35 M sodium hydrogen phosphate buffer (45 mL total volume,
pH 7.3) was heated to 50 °C for 60 min (*Acidovorax facilis* 72W) or 12 min
(*E. coli* transformant SS1001), then added to the carrageenan solution at 55-56 °C
with stirring. The cell/carrageenan mixture was immediately added slowly to
15 450 mL of soybean oil at 50 °C with stirring using an overhead stirrer. After
cell/carrageenan droplets of the desired size were produced in the oil by
controlling the stirring rate, the temperature of the oil was reduced to 35 °C to gel
the droplets, and the oil decanted from the resulting beads, which were washed
with 0.10 M potassium bicarbonate buffer (pH 7.3). A 20-gram portion of the
20 beads were resuspended in 48.8 mL of 0.10 M potassium bicarbonate buffer
(pH 7.3), 0.25 g of 25 wt% glutaraldehyde in water was added, and the beads
mixed for 1.0 h at 25 °C. To the mixture was then added 1.0 g of 12.5 wt%
polyethylenimine (BASF Lupasol® PR971L, average Mw ca. 750,000) in water
and the beads mixed for an additional hour at 25 °C. The crosslinked beads were
25 then washed with 50 mL of 0.30 M ammonium bicarbonate (pH 7.3) at 25 °C and
stored in this same buffer at 5 °C.

EXAMPLE 9

Comparison of Carrageenan-Immobilized *Acidovorax facilis* 72W and *E. coli*

Transformant SS1001 Cells as Catalyst for Production of

4-Cyanopentanoic Acid Ammonium Salt

30 In a typical reaction, 16.5 g of immobilized cell/carrageenan beads were
placed into a 125 mL jacketed reaction vessel that was temperature-controlled at
30 °C with a recirculating temperature bath. To the reaction vessel was added
69.25 mL of water and 14.25 mL (13.54 g, 1.25 M) of 2-methylglutaronitrile, and
35 the mixture stirred at 30 °C. Samples (0.100 mL) were mixed with 0.400 mL of
water, then 0.360 mL of the diluted sample was mixed with 0.040 mL of 0.75 M
N-methylpropionamide in water and 0.020 mL of 6.0 N HCl. The resulting
mixture was filtered (0.22 µm) and the filtrate analyzed by HPLC for

2-methylglutaronitrile, 4-cyanopentanoic acid and 2-methylglutaric acid. The rates of production of 4-cyanopentanoic acid when using carrageenan-immobilized *Acidovorax facilis* 72W and *E. coli* transformant SS1001 cells were 184 mM/h and 310 mM/h, respectively. At complete conversion of 2-methylglutaronitrile, each catalyst produced 4-cyanopentanoic acid ammonium salt and 2-methylglutaric acid diammonium salt in 98.7 % and 1.3 % yields, respectively.

EXAMPLE 10

Immobilization of *E. coli*

Transformant SW91 Cells in Calcium Alginate

Into a 100 mL media bottle (equipped with magnetic stir bar and containing 22.9 g of distilled, deionized water at 50 °C) was slowly added 1.10 g of FMC BioPolymer Protanal® LF 10/60 alginate with rapid stirring. The mixture was heated to 75-80 °C with rapid stirring until the alginate was completely dissolved, and the resulting solution cooled to 25 °C in a water bath. A suspension of *E. coli* transformant SW91 (50 % wet cell weight, 11.5 % dry cell weight) in 0.15 M sodium acetate buffer (16 mL total volume, pH 7.0) was added to the alginate solution at 25 °C with stirring. The cell/alginate mixture was added dropwise by syringe to 213 mL of 0.20 M calcium acetate buffer (pH 7.0) at 25 °C with stirring. After stirring for 2 h, the buffer was decanted from the resulting beads, which were resuspended in 84 mL of 0.20 M calcium acetate buffer (pH 7.0) at 25 °C. With stirring, 0.88 g of 25 wt% glutaraldehyde in water was added and the beads mixed for 1.0 h at 25 °C. To the mixture was then added 3.5 g of 12.5 wt% polyethylenimine (BASF Lupasol® PR971L, average Mw ca. 750,000) in water and the beads mixed for an additional 1 h at 25 °C. The crosslinked beads were then washed twice with 84 mL of 0.20 M calcium acetate buffer (pH 7.0) at 25 °C and stored in this same buffer at 5 °C.

EXAMPLE 11

Calcium Alginate-Immobilized *E. coli* Transformant SW91 Cells as Catalyst for Production of 4-Cyanopentanoic Acid Ammonium Salt

Into a 125 mL jacketed reaction vessel (temperature-controlled at 30 °C with a recirculating temperature bath) was placed 16.5 g of *E. coli* SW91/alginate beads prepared as described in Example 10. To the reaction vessel was added 68.25 mL of water, 1.0 mL of 0.20 M calcium acetate buffer (pH 7.0), and 14.25 mL (13.54 g, 1.25 M) of 2-methylglutaronitrile, and the mixture stirred at 30 °C. Samples (0.100 mL) were mixed with 0.400 mL of water, then 0.360 mL of the diluted sample was mixed with 0.040 mL of 0.75 M N-methylpropionamide in water and 0.020 mL of 6.0 N HCl. The resulting mixture was filtered (0.22 µm) and the filtrate analyzed by HPLC for 2-methylglutaronitrile,

4-cyanopentanoic acid and 2-methylglutaric acid. The rate of production of 4-cyanopentanoic acid was 739 mM/h, and the reaction was complete in less than 2.5 h. At complete conversion of 2-methylglutaronitrile, the yields of 4-cyanopentanoic acid ammonium salt and 2-methylglutaric acid diammonium salt were 98.5 % and 1.5 % yields, respectively.

At the end of the reaction the product mixture was decanted from the catalyst beads, which were reused in an additional five consecutive batch reactions as described above. The rate of production of 4-cyanopentanoic acid in reaction six was 721 mM/h, and the reaction was complete in less than 2.5 h. At complete conversion of 2-methylglutaronitrile, the yields of 4-cyanopentanoic acid ammonium salt and 2-methylglutaric acid diammonium salt were 98.0 % and 2.0 % yields, respectively.

EXAMPLE 12

Total Turnover Number (TTN) for Immobilized

Acidovorax facilis 72W Cell Nitrilase

Into a 125 mL jacketed reaction vessel (temperature-controlled at 25 °C) was placed 16.5 g of immobilized *Acidovorax facilis* cell catalyst (prepared as described in Example 8). To the reaction vessel was added 72.1 mL of water and 11.4 mL (10.83 g, 1.0 M) of MGN, and the mixture stirred at 25 °C. Samples (0.100 mL) were mixed with 0.400 mL of water, then 0.360 mL of a diluted sample was mixed with 0.040 mL of 0.75 M N-methylpropionamide in water and 0.020 mL of 6.0 N HCl. The resulting mixture was filtered (0.22 µm) and the filtrate analyzed by HPLC. After complete conversion of MGN, the product mixture and catalyst were decanted to a tared 250 mL beaker and the aqueous product mixture decanted from the catalyst. The remaining catalyst and product mixture heel was weighed and the weight recorded, then water was added to the catalyst to a final total weight (water and catalyst) of 88.6 g. The catalyst suspension was transferred back to the reaction vessel, 11.4 mL of MGN added, and the reaction repeated.

A total of sixty-seven consecutive batch reactions with catalyst recycle were run. There was no measurable loss of catalyst bead weight over the course of the sixty-seven recycle reactions, and 1001 g 4-CPA/g dcw *Acidovorax facilis* 72W cells were produced. The initial reaction rate was 142 mM 4-CPA/h, which corresponds to 237 IU of nitrilase activity, or 6.76 mg (1.7×10^{-7} mole) of 72W nitrilase. The amount of 4-CPA produced in 67 consecutive batch reactions was 6.6 moles, thus the total turnover number (TTN = moles product per moles enzyme) was $6.6/1.7 \times 10^{-7}$, or 3.9×10^7 TTN.

EXAMPLE 13

Expression of *Acidovorax facilis* 72W nitrilase coding sequence in *Pichia pastoris*

A synthetic nitrilase gene (SEQ ID NO:16) (which encodes a protein sequence identical to that found in *Acidovorax facilis* 72W (SEQ ID NO:14)) was constructed using sixteen 90 bp oligomers (SEQ ID NOS:17-32) and the technique of PCR-mediated overlap extension. The synthetic gene optimizes the codon usage found in *Pichia pastoris*, which is considerably different from that found in the *A. facilis* 72W nitrilase gene sequence. After confirming by nucleotide sequencing, the synthetic nitrilase gene is subcloned into pGAPZA and into pPICZA (Invitrogen, San Diego, CA) at the *EcoRI* site. pGAPZA uses the constitutive *P. pastoris* GAP (glyceraldehyde-3-phosphate dehydrogenase) promoter to drive expression of foreign genes; pPICZA uses the methanol inducible *P. pastoris* AOXI (alcohol oxidase I) promoter to drive expression of foreign genes. Plasmids pGAP::nit and pAOX::nit are used to transform *P. pastoris* GS115 (Invitrogen) to zeocin resistance by spheroplast transformation essentially as described (Cregg et al., *Mol. Cell Biol.* (1985) 5:3376-3385). After appropriate growth and induction (only pAOX::nit requires induction), transformants producing nitrilase are identified by SDS-PAGE protein analysis of cells extracts prepared essentially as described (Sreekrishna et al., *Biochem* (1989) 28:4117-4125), and by enzyme activity assay as previously described (Examples 6 and 7).

EXAMPLE 14

Determination of *Acidovorax facilis* 72W nitrilase as % of total soluble protein

A crude extract of *Acidovorax facilis* 72W was prepared by passage of a 25 wt% cell suspension through a french press as previously described (Example 1). Protein components in an aliquot of the soluble protein fraction of the crude extract were separated by gel electrophoresis (SDS-PAGE) performed under reducing conditions (5 % β -mercaptoethanol) on a 10-15 % SDS polyacrylamide gel. Following electrophoresis, the gel was treated for 15 min at 25 °C with a solution composed of 0.2 % Coomassie Brilliant Blue dye, 30 % methanol, and 10 % acetic acid. Protein bands were visualized in the gel following de-staining with a solution composed of 30 % methanol and 10 % acetic acid. The protein bands in the gel were then integrated using an LKB Ultrosan XL Enhanced Laser Densitometer. The nitrilase band having a subunit MW of ca. 40 kd represented 3.4 % of the total soluble protein detected on the gel.

EXAMPLE 15

Construction of SS1011 [MG1655(DE3):pnitex2] and its nitrilase activity

- λDE3 prophage was site-specifically integrated into the chromosome of *E. coli* strain MG1655 (ATCC 47076) to yield strain MG1655(DE3). λDE3
- 5 lysogenization kit (Catalog No 69734-3, Novagen, Inc. Madison, WI) was used for this purpose according to manufacturer's instructions. MG1655(DE3) was transformed with plasmid pnitex2 described in Example 6 to yield SS1011. Strains SS1011 and SS1001 were grown in LB medium for 16-17 h. Whole cell nitrilase activity as shown in Table 5 was determined using a microliter plate-
- 10 based spectrophotometric assay performed at 35 °C that measures the hydrolysis of benzonitrile (9.5 mM, 0.1 M phosphate buffer, pH 7.0) to produce benzoic acid as indicated by the increase in absorption at 245 nm. The nitrilase activity units (IU) determined using this assay are typically 5-6 fold higher than those determined using the previously described methylglutaronitrile assay due to a combination of
- 15 higher assay temperature (35 °C vs. 25 °C) and the relatively higher substrate specificity of the enzyme for benzonitrile.

Table 5: Nitrilase activity in *E. coli* transformants SS1001 and SS1011 determined

by assay of hydrolysis of benzonitrile

Transformant Catalyst	Nitrilase Activity (IU/g dry cell weight)
<i>E. coli</i> SS1011 (MG1655(DE3): pnitex2)	3652
<i>E. coli</i> SS1001 (BL21(DE3): pnitex2)	4500
<i>Acidovorax facilis</i> 72W (ATCC 55746)	1491